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GRANT NUMBER: DAMD17-94-J-4193

TITLE: Protein Kinase C Processes and Their Relation to
Apoptosis in Human Breast Carcinoma Cells

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REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1996	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 95 - 31 Aug 96)	
4. TITLE AND SUBTITLE Protein Kinase C Processes and Their Relation to Apoptosis in Human Breast Carcinoma Cells			5. FUNDING NUMBERS DAMD17-94-J-4193	
6. AUTHOR(S) John S. Lazo, Ph.D. Robert L. Rice				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pittsburgh School of Medicine Pittsburgh, Pennsylvania 15261			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
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14. SUBJECT TERMS Breast cancer Cdc25 phosphatases, Combinatorial Chemistry, Inhibitor			15. NUMBER OF PAGES 54	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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Robert A. Rier
PI - Signature

10/11/96
Date

Title: Function of Cdc25 Phosphatase in the Development of Human Breast Cancer

Abstract:

It was reported previously that the specific aim of screening agents that would induce apoptosis in the human breast carcinoma cell line MDA-MB-231 was completed, and that calyculin A was the most effective compound tested. Since then Cdc25 phosphatases were reported as possible key oncogenes in human breast carcinoma¹. In light of our results with calyculin A and an ongoing collaboration with Dr. Peter Wipf in the department of Chemistry at the U. of Pittsburgh in evaluating a combinatorial library of calyculin A analogues, it was decided that a more promising focus for the project would be the biochemical basis for the oncogenic actions of Cdc25 phosphatases in human breast carcinoma.

At this time we have examined 18 compounds and discovered a Cdc25 phosphatase inhibitor, called 1f, that selectively inhibits Cdc25A,B, and C, has antiproliferative activity against and causes a G₁ block in MDA-MB-231 cells. The pure form of this compound and two other compounds which selectively inhibit Cdc25 phosphatases have been made and also demonstrate an equal if not better inhibition of Cdc25 phosphatases as their combinatorial form.

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F. Introduction

1. Background

I. Solid Tumors and the cell cycle

Solid tumors such as breast cancer are almost completely refractory to current chemotherapeutic agents, and new drugs that exploit recent biological discoveries are necessary. It is now generally accepted that nearly all forms of neoplasia have altered cell cycle control⁵ frequently due to enhanced or altered mitogenic signaling, reduced cell cycle checkpoints, or diminished apoptosis.

Cdc25 phosphatases have been proposed as key oncogenes in human breast carcinoma. In human primary breast cancers tested by Galaktionov et al. overexpression of Cdc25B phosphatase was detected in 32% of patients with axillary node negative invasive breast cancer. Human Cdc25A & B phosphatases also cooperated with Ha-Ras^{G12V} and Cdc25A cooperated with Rb^{-/-} in the oncogenic transformation of mouse embryonic fibroblasts (MEF)¹. Therefore, phosphorylation signaling pathways involving Cdc25 are potentially rewarding sites in the search for new chemotherapeutic agents and a better understanding of oncogenesis.

II. Cdc25 phosphatases are dual specificity phosphatases

The first dual specificity phosphatase (DSPase) to be discovered, VH1, corresponded to the H1 open reading frame in the vaccinia virus⁶. Several additional members of this family have been discovered such as MAPK phosphatases and, most notably, Cdc25 phosphatases. DSPases can dephosphorylate phosphotyrosine, phosphothreonine, and phosphoserine residues. DSPases in general appear to have a marked preference for protein kinases that are phosphorylated on tyrosine and threonine residues that are in close proximity. This is especially true for certain cyclin dependent kinases. The

substrate motif for Cdc25 phosphatases is -pTpY-³. The novel DSPase class, especially Cdc25 phosphatases, are emerging as important regulators of the cell cycle.

III. Cell Cycle and Cdc25 phosphatases

The current model of cell cycle control maintains that the transition between different cell cycle phases are regulated at checkpoints. The integrity of these checkpoints is considered vital in avoiding malignancy. Progression of eukaryotic cells through the cell cycle is controlled in part by the activity of cyclin dependent kinases (cdk) and by protein phosphatases such as Cdc25 phosphatases through a complex phosphorylation cascade (Figure 1)⁸.

Cdc25 phosphatases are key regulators in the progression of eukaryotic cells from one phase of the cell cycle to the next. The best studied cell cycle event is the transition from G₂ to M phase, in which a nuclear protein complex that contains the catalytic subunit Cdc2 and the regulatory subunit cyclin B has a crucial role. In mammalian cells the activity of Cdc2 is controlled by phosphorylation at three sites^{9,10}: T¹⁶¹, T¹⁴, and Y¹⁵. Phosphorylation of T¹⁶¹ is absolutely required for activation, while phosphorylation of T¹⁴ and Y¹⁵ by wee1 or mik1 inhibits Cdc2 activity. Cdc25C is the most likely human isoform responsible for dephosphorylating Cdc2 at T¹⁴ and Y¹⁵ and regulates the G₂/M transition of the cell cycle^{9,10}. Other than their oncogenic properties the precise biological functions and role in the cell cycle of the Cdc25A & B phosphatases in the cell cycle are still poorly defined. Cdc25A appears to participate in controlling the cell cycle either at the G₂/M or, more likely, the G₁/S transition where it can be phosphorylated and activated by the cdk2-cyclin E complex^{3,4,10}. Cdc25B has recently been proposed to participate in regulating the G₂/M transition². Cdc25 phosphatases in general have an active role in regulating the checkpoints of the cell cycle.

It is known that the phosphatase catalytic domains of Cdc25A and Cdc25B are located within the carboxyl terminus¹¹. Although it is generally assumed that the

biological effects of Cdc25 phosphatases are due to intrinsic phosphatase activity, this has been difficult to establish firmly. Attempts to use obvious genetic approaches, such as deletion mutagenesis, have revealed gain of function or new protein-protein interactions for the mutants with deleted phosphatase activity making conclusions about phosphatase activity and function difficult¹¹. Consequently, there is considerable interest in a more pharmacological approach to generate selective antagonists of the phosphatase activity of DSPases. Inhibitors of Cdc25C have potential as selective disrupters of the G₂/M transition, and thus, could be unique antimitotic agents. The Cdc25A and B isoforms could control transition through other domains of the cell cycle and selective antagonists of these DSPases could provide novel cell cycle inhibitors. These inhibitors also have the potential as apoptotic agents *per se* or enhancers of anticancer drug-induced apoptosis¹².

2. Technical Objectives

- (1) Characterize the ability of a small library of novel, nonpeptidic, small-molecules to inhibit Cdc25 phosphatase *in vitro* using recombinant proteins
- (2) Examine the selectivity of this inhibition among phosphatases
- (3) Determine the antiproliferative and antiphosphatase activity of these novel compounds with intact cells.

G. Body of Proposal

1. Experimental Methods

Cell Culture. Human MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection at passage 28 and were maintained for no longer than 20 passages. The cells were grown in RPMI-1640 supplemented with 1% penicillin (100 $\mu\text{g/mL}$) and streptomycin (100 $\mu\text{g/mL}$), 1% L-glutamate, and 10% fetal bovine serum in a humidified incubator at 37⁰ C under 5% CO₂ in air. Cells were routinely found free of mycoplasma. To remove cells from the monolayer for passage or flow cytometry, we washed them two times with phosphate buffer and briefly (< 3 min) treated the cells with 0.05% trypsin/2 mM EDTA at room temperature. After the addition of at least two volumes of growth medium containing 10% fetal bovine serum, the cells were centrifuged at 1,000 x g for 5 min. Compounds were made into stock solutions using DMSO, and stored at -20⁰C. All compounds and controls were added to make a final concentration of 0.1-0.2% (v/v) of the final solution for experiments.

Cell proliferation assay. The antiproliferative activity of newly synthesized compounds was determined by our previously described method (Lazo, J.S., Kondo, Y., Dellapiazza, D., Michalska, A.E., Choo, K.H. Andy and Pitt, B.R. Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. J. Biol. Chem. 270:5506-5510, 1995). Briefly, cells (6.5×10^3 cells/cm²) were plated in 96 well flat bottom plates for the cytotoxicity studies and incubated at 37⁰C for 48 h. The plating medium was aspirated off 96 well plates and 200 μL of growth medium containing drug was added per well. Plates were incubated for 72 h, and then washed 4x with serum free medium. After washing, 50 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide solution (2 mg/mL) was added to each well, followed by 150 μL of complete growth medium. Plates were then incubated an additional 4 h at 37⁰C. The solution was aspirated off, 200 μL of DMSO added, and the plates shaken for 30 min at room

temperature. Absorbance at 540 nm was determined with a Titertek Multiskan Plus plate reader. Biologically active compounds were tested at least 3 independent times.

Measurement of cell cycle kinetics. Cells ($6.5 \times 10^5/\text{cm}^2$) were plated and incubated at 37°C for 48 h and then treated with growth medium for 72 h containing a concentration of C3B that caused approximately 50% growth inhibition (88 μM). Untreated cells at a similar cell density were used as control populations. Single cell preparations were fixed in ice-cold 1% paraformaldehyde, centrifugation at $1,000 \times g$ for 5 min, resuspended in Puck's saline, centrifuged, and resuspended in ice-cold 70% ethanol overnight. The cells were removed from fixatives by centrifugation ($1,000 \times g$ for 5 min) and stained with a 5 $\mu\text{g}/\text{mL}$ propidium iodide and 50 $\mu\text{g}/\text{mL}$ RNase A solution. Flow cytometry analyses were conducted with a Becton Dickinson FACS Star. Single parameter DNA histograms were collected for 10,000 cells, and cell cycle kinetic parameters calculated using DNA cell cycle analysis software version C (Becton Dickinson). Experiments were performed at least 3 independent times.

Dual Specificity Phosphatase Assay. The activity of the dual specificity phosphatases (GST-fusion proteins made by previously described methods) were measured with 3,6 fluorescein diphosphatase (Molecular Probes, Inc., Eugene, Or) as a substrate in a 96-well microtiter plates. The final incubation mixture (150 μl) comprised 30 mM Tris (pH = 8.5), 75 mM NaCl, 0.67 mM EDTA, 0.033% BSA, 1 mM DTT and 20 μM 3,6 fluorescein diphosphatase. Inhibitors were resuspended in DMSO, which was also used as the vehicle control. Reactions were initiated by adding 12 μg of fusion protein and incubated at ambient temperature for one hour. Fluorescence emission from the product was measured with Perseptive Biosystems Cytofluor II (excitation filter, 485/20;emission filter, 530/30) (Framingham, MA).

2. Results

After screening several compounds from a combinatorial library developed in collaboration with Dr. Peter Wipf, we discovered compounds that could inhibit Cdc25 phosphatases. Characterization of these compound was initially evaluated by their ability to inhibit tyrosine dephosphorylation. The initial dephosphorylation step for DSPases is the removal of the Y phosphate. Thus we used a sensitive microtiter assay for Y dephosphorylation that has already been established¹³ as our initial characterization of these compounds ability to inhibit Cdc25 phosphatases (Figure 2). We have also investigated whether these compounds inhibits DSPases specifically and Cdc25 phosphatases in particular by studying their ability to inhibit serine/threonine phosphatases such as PP1, PP2A and PP3 by the classic phosphate release assay and other dual specificity phosphatases such as CL100, a MAPK phosphatase. Compounds 1c, 1e, and 1f appear to be the strongest inhibitors of Cdc25 phosphatases. Evidence that indicates compounds 1c, 1e and 1f inhibit Cdc25 phosphatases include: a concentration response curve (Figure 3) where pure 1e and 1f demonstrate an $IC_{50} \sim 10 \mu M$ and 1c demonstrates an $IC_{50} \sim 100 \mu M$ for both GST-Cdc25A and GST-Cdc25B, a $\sim 50\%$ inhibition of Cdc25C at $100 \mu M$ by 1c, 1e and 1f and a lack of inhibition of serine/threonine phosphatases such as PP1 and PP2A (courtesy of Dr. Alton Boynton) and MAPK phosphatase CL100 by these compounds.

The effects of inhibiting Cdc25 phosphatases in a human breast carcinoma cell line was studied in MDA-MB-231 cells. This cell line was chosen because it expresses Cdc25A & B (data not shown) and has a mutation in codon 13 giving rise to a Ki-ras^{G14R} mutated allele¹⁵. This cell line has two of the characteristics necessary for Cdc25 phosphatase related transformation. This makes it an excellent model for testing the effects of inhibiting Cdc25 phosphatase in a cancer cell line.

Only two compounds had any antiproliferative effect against MDA-MB-231 cells (Figure 4), and only compound 1f demonstrated more than a 50% inhibition of growth among the two. Therefore, we first evaluated the antiproliferative activity of combinatorial 1f in MDA-MB-231 cell lines in a concentration-response study using our previously described MTT microtiter assay^{16,17}. We also characterized the cell cycle phase-specificity of the inhibitor 1f. Asynchronous growing MDA-MB-231 cells were treated with 1f to investigate gross cell cycle perturbations using flow cytometry. The biological effect of 1f in these include: antiproliferative activity with an $IC_{50} \sim 100 \mu M$ in MDA-MB-231 cells and a definite G_1 block upon treatment with $88 \mu M$. (Figure 4)

H. Conclusions

It was reported previously that the specific aim of screening agents that would induce apoptosis in the human breast carcinoma cell line MDA-MB-231 was completed, and that calyculin A was the most effective compound tested. Since then Cdc25 phosphatases were reported as possible key oncogenes in human breast carcinoma¹. In light of our results with calyculin A and an ongoing collaboration with Dr. Peter in evaluating a combinatorial library of calyculin A analogues, it was decided that a more opportune focus for the project would be the biochemical basis for the oncogenic actions of Cdc25 phosphatases in human breast carcinoma.

At this time we have examined 18 compounds and discovered a Cdc25 phosphatase inhibitor, called 1f, that selectively inhibits Cdc25A,B, and C and has antiproliferative activity against and causes a G_1 block in MDA-MB-231 cells. The pure form of this compound and two other compounds which selectively inhibit Cdc25 phosphatases have been made and also demonstrate an equal if not better inhibition of Cdc25 phosphatases as their combinatorial form. This accomplishes most of the first technical objective by demonstrating the ability of a small library of novel, nonpeptidic, small-molecules to inhibit Cdc25 phosphatase *in vitro* using recombinant proteins. We are also well on the way to

accomplishing the second objective of examining the selectivity of this inhibition among phosphatases. We have already done preliminary studies with Cdc25 A,B, &C phosphatases, the serine/threonine phosphatases PP1,PP2, and PP3 courtesy of Dr. Alton Boyington and the MAPK phosphatase CL100. We also have the plasmids for the VHR DSPase and the rat tyrosine phosphatase homologue of human PTP1B courtesy of Dr. Jack E. Dixon. This lays out the ground work for further kinetic studies with these compounds as outlined in the statement of work.

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J. Addendum

1. Statement of Work

Function of Cdc25 Phosphatase in the Development of Human Breast Cancer

Task I. Characterize the ability of a small library of novel, nonpeptidic, small-molecules to inhibit Cdc25 phosphatase *in vitro* using recombinant proteins. Months 1-15

1. Evaluation for DSPase activity
 - a) Microtiter assay for dephosphorylation to evaluate initial library elements using recombinant Cdc25A,B, & C currently being made in our laboratory.
 - b) Complete kinetic analysis will be done on all highly active compounds

Task II. Examine the selectivity of this inhibition among phosphatases. Months 15-24

1. Primary evaluation for DSPase activity
 - a) Microtiter assay for dephosphorylation to evaluate initial library elements using recombinant Cdc25A,B, & C and CL100 currently being made in our laboratory and VHR, PTP1B, PP1, PP2A and PP3.
 - b) Complete kinetic analysis will be done on all highly active compounds.
2. Secondary evaluation for DSPase activity
 - a) Primary assay permits rapid analysis, but the artificial substrate used may not fully emulate enzyme-substrate interactions. Therefore, once we have identified an active compound, we will use phosphopeptide substrates to determine all four possible phosphorylation states of the peptide in the presence and absence of the active compound by HPLC assay.

Task III. Determine the antiproliferative and antiphosphatase activity of these novel compounds with intact cells. Months 22-36.

1. We will use the previously described MTT microtiter assay to determine the antiproliferative and cytotoxicity activity of this initial combinatorial library using MDA-MB-231 cells or mouse

embryo fibroblasts (H-Ras^{G12V+}/Cdc25A; H-Ras^{G12V+}/Cdc25B;
H-Ras^{G12V+}/Cdc25C)

- a) Concentration-response curve
- b) Time studies

2. Training

In fulfillment of my doctoral training, I have completed all required course work and comprehensive exam, and I am currently a Ph.D. candidate. I have had my first thesis committee this July 8, 1996, and plan to have my second meeting this mid-October, 1996 to finalize my thesis goals with my committee. I participate in the Predoctoral training Program in Breast Cancer Biology and Therapy, and regularly attend seminars.

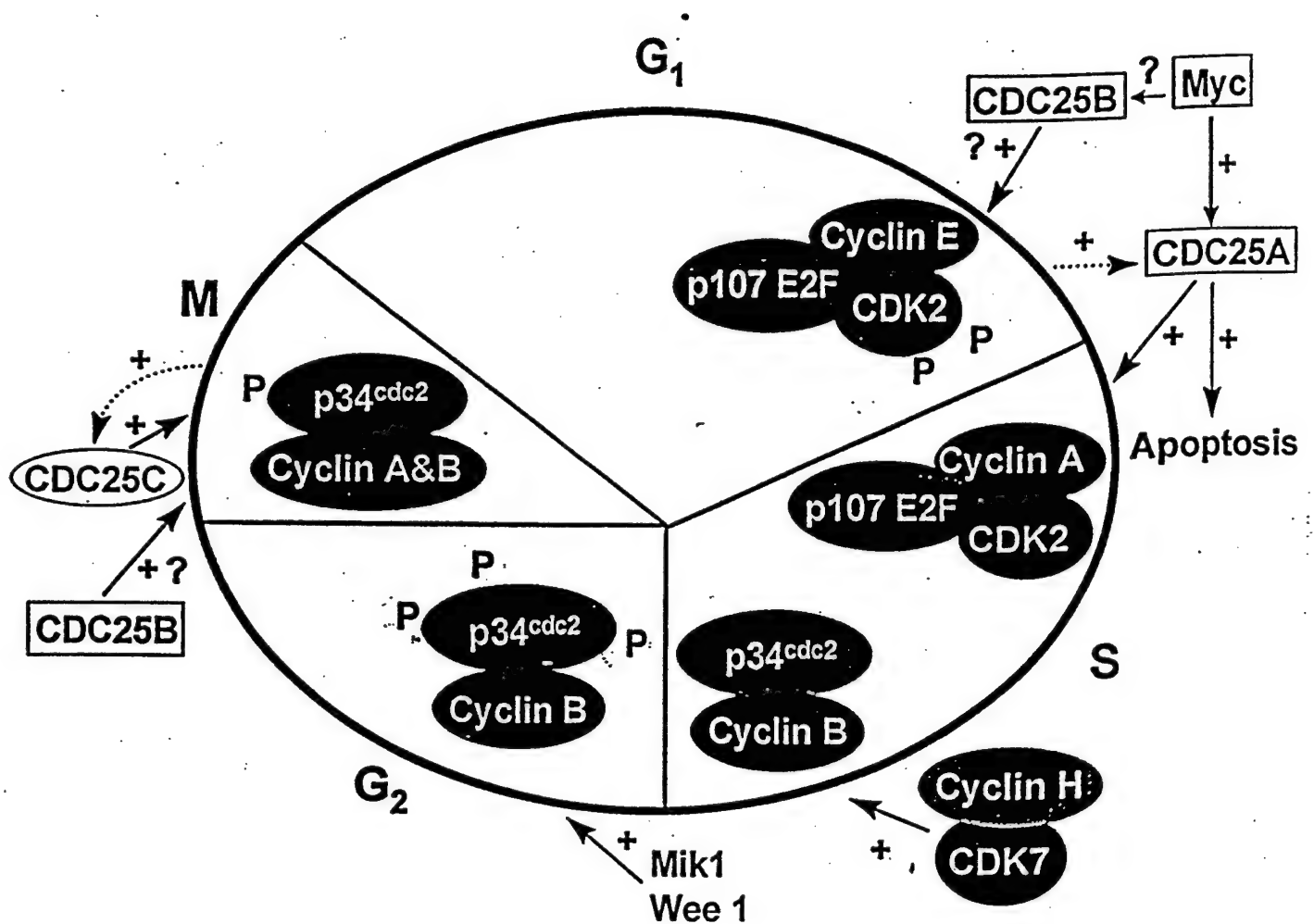


Figure 1. Simplified schematic of the proposed sites of action of Cdc25 isoforms. Effects on Cdc25 by CDK are indicated with dotted lines. Oncogenic Cdc25 proteins are signified by a box.

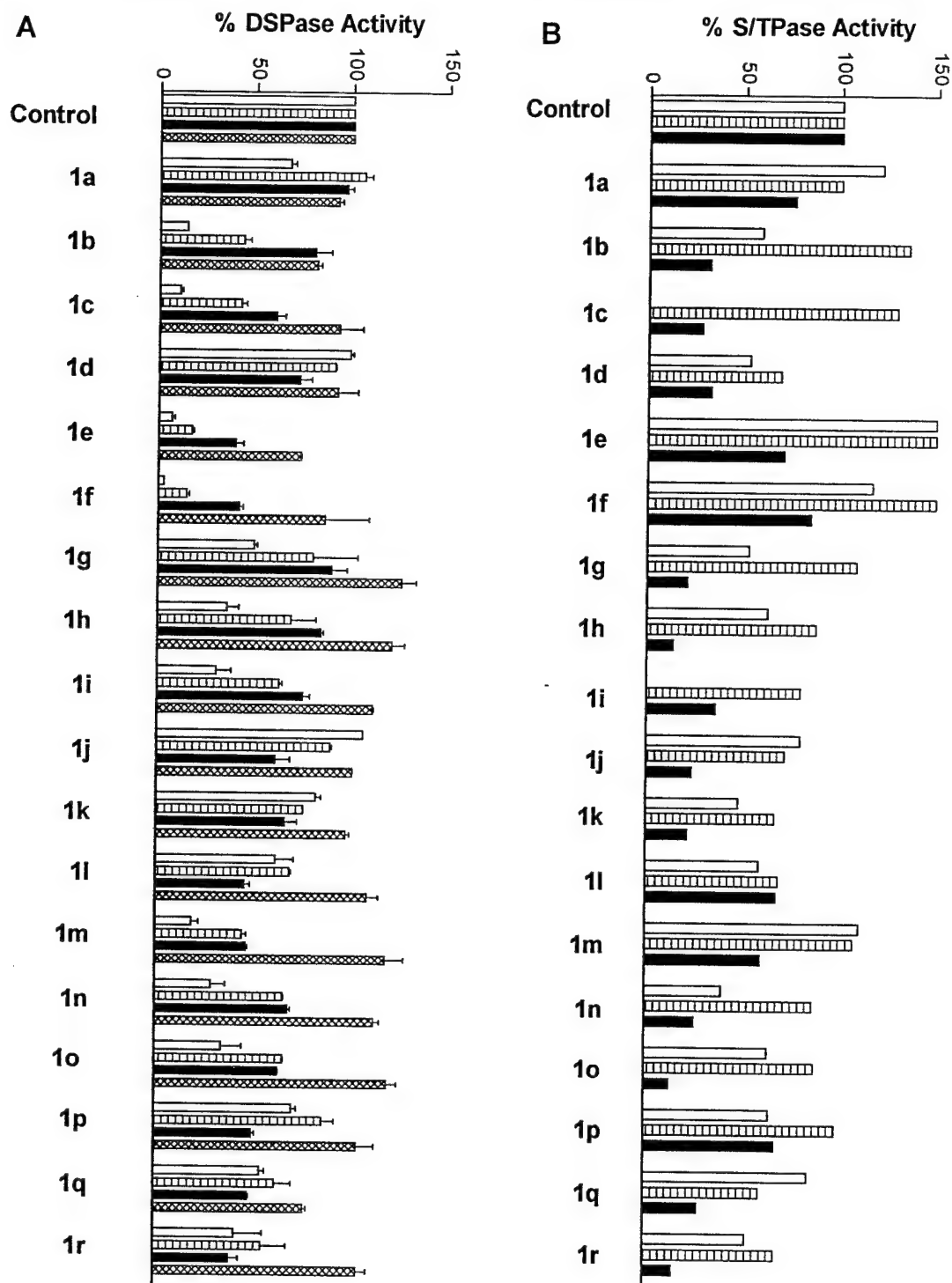
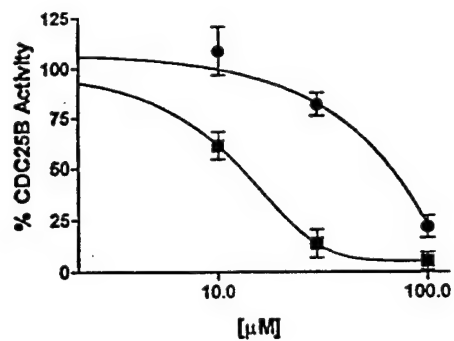
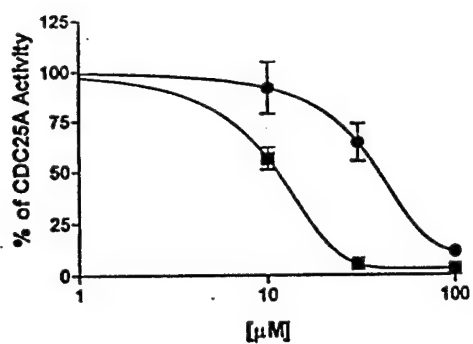
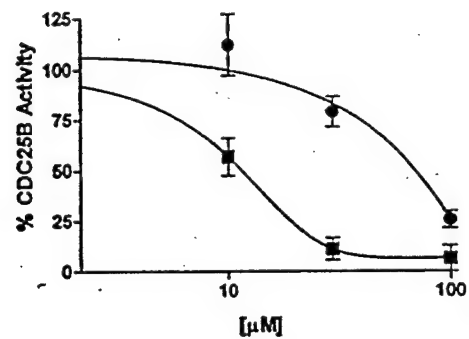
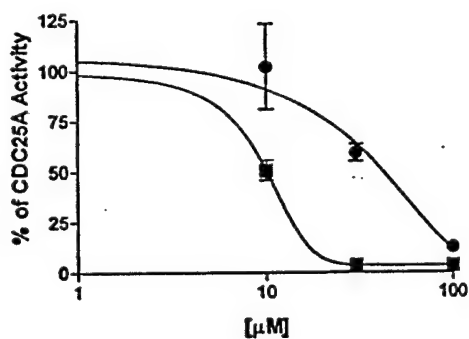


Figure 2. Antiphosphatase activity of combinatorial compounds. Panel A. Inhibition of DSPases Cdc25A (open bars), Cdc25B (horizontal bars), Cdc25C (black bars) or CL100 (hatched bars) activity after incubation with 100 μ M for 60 min. each value is the mean result of 2 independent experiments. Bars=SD. Panel B. Inhibition of PSTPase PP1 (open bars), PP2A (horizontal bars) or PP3 (black bars) by combinatorial compounds.

A



B



C

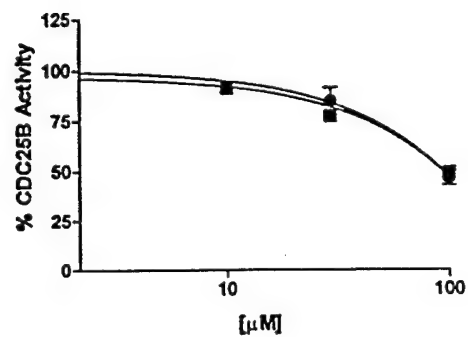
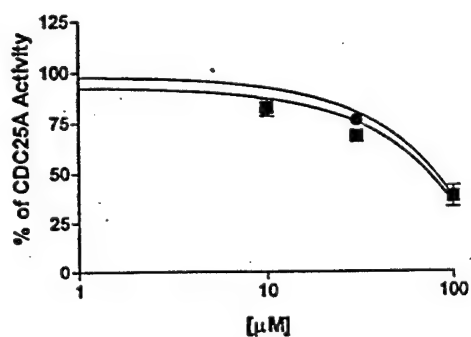


Figure 3. Concentration-dependent inhibition of Cdc25A and B by combinatorial and pure calyculin A analogues. Compounds that were generated by combinatorial methods are indicated in circles and pure compounds are indicated with squares. Panel A. **1f** Panel B. **1e** Panel C. **1c** Each determination is for three independent determination. Bars = SEM.

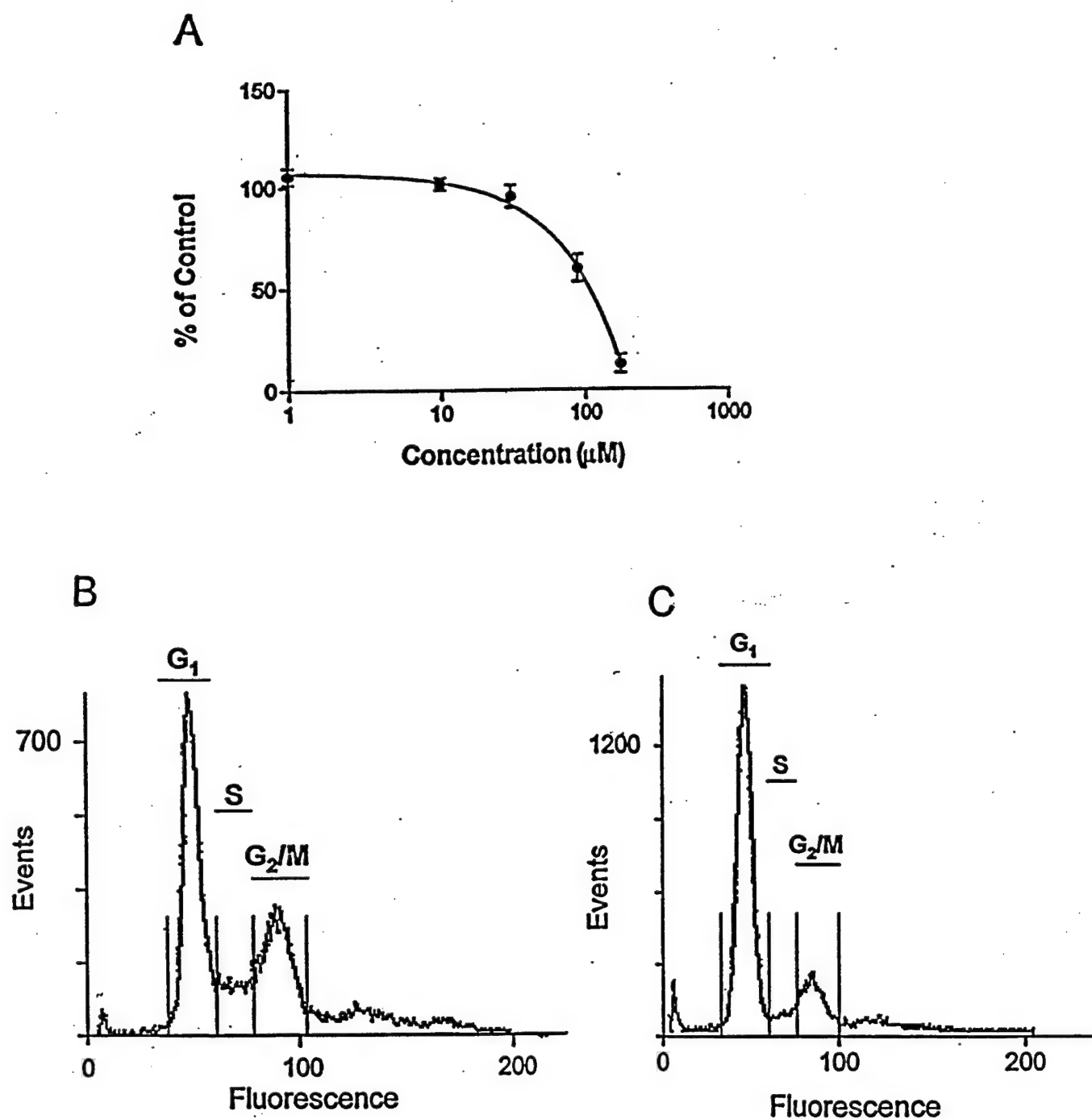
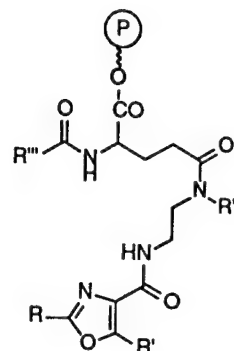


Figure 4. Antiproliferative and flow cytometry results with 1f. Panel A. Concentration-response of the antiproliferative effects of 1f against MDA-MB-231 breast cancer cells. Cells were incubated with 88 μM 1f for 72-h and the cell number determined with an MTT assay. B. Cell cycle distribution of untreated MDA-MB-231 cells. Panel C. Cell cycle distribution of MDA-MB-231 cells 72 h after 88 μM 1f.

Combinatorial Synthesis and Biological Evaluation of a Library of Small-Molecule Ser/Thr-Protein Phosphatase Inhibitors

P. Wipf,* A. Cunningham, Department of Chemistry,
R. L. Rice, and J. S. Lazo,* Department of Pharmacology,
University of Pittsburgh, Pittsburgh, PA 15260.

Based on functional groups present in natural product serine/threonine protein phosphatase inhibitors, we have designed a pharmacophore model and demonstrated the feasibility of a solid-phase combinatorial chemistry approach for the preparation of functional analogs. Preliminary biological testing of 18 structural variants exhibited a concentration-dependent inhibition in proliferation of MDA-MB-231 cells and flow cytometry data confirmed blockage in cell cycle progression at the G1 checkpoint.



Combinatorial Synthesis and Biological Evaluation of a Library of Small-Molecule Ser/Thr-Protein Phosphatase Inhibitors

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Abstract: In eukaryotes, phosphorylation of serine, threonine, and tyrosine residues on proteins is a fundamental posttranslational regulatory process for such functions as signal transduction, gene transcription, RNA splicing, cellular adhesion, apoptosis, and cell cycle control. Based on functional groups present in natural product serine/threonine protein phosphatase (PSTPase) inhibitors, we have designed pharmacophore model **1** and demonstrated the feasibility of a combinatorial chemistry approach for the preparation of functional analogs of **1**. Preliminary biological testing of 18 structural variants of **1** has identified two compounds with growth inhibitory activity against cultured human breast cancer cells. *In vitro* inhibition of the PSTPase PP2A was demonstrated with compound **1d**. Using flow cytometry we observed that compound **1f** caused prominent inhibition in the G1 phase of the cell cycle. Thus, the combinatorial modifications of the minimal pharmacophore **1** can generate biologically interesting antiproliferative agents.

Introduction

Many eukaryotic cell functions, such as signal transduction, cell adhesion, gene transcription, RNA splicing, apoptosis, and cell proliferation, are controlled by protein phosphorylation, which is regulated by the dynamic relationship between both kinases and phosphatases.¹ Indeed, the principal role of many second messengers is to modulate kinase selectivity. In an effort to intervene early in the initiation stage of cellular events and in recognition of the tumor promoting effects of phorbol ester based protein kinase C activators, the lion's share of synthetic chemistry research in this area has focused on protein kinases.² However, there is substantial recent biological evidence for the multiple regulatory functions of protein phosphatases and a clear link between phosphatase inhibition and apoptosis.^{3,4,5,6,7,8,9}

Besides some minor phosphorylation of histidine, lysine, arginine, and, in bacteria, aspartate, most eukaryotic amino acid phosphate derivatives are found on serine, threonine, and tyrosine protein residues. Generally, the primary characterization of phosphatases follows these structural guidelines: Ser/Thr protein phosphatases (PSTPases), Tyr protein phosphatases (PTPases), and dual-specificity phosphatases (DSPases).³

PSTPases have been classified according to their substrate specificity, metal ion dependence and sensitivity to inhibition (Table 1).^{10,11} cDNA cloning has revealed at least 40 different enzymes of this type. In addition to proteins (Inhibitor-1, Inhibitor-2, DARPP-32, NIPP-1),⁴ several (mostly marine) toxins have been identified as potent inhibitors (Figure 1).¹²

Okadaic acid is produced by several species of marine dinoflagellates and reversibly inhibits the catalytic subunits of the PSTPase subtypes PP1, PP2A, and PP3.⁴ SAR studies showed that the carboxyl group as well as the four hydroxyl groups were important for activity.^{13,14} Calyculin A was identified as a cytotoxic component of the marine sponge *Discodermia calyx*. It has an extremely high affinity to PP1, PP2A, and PP3 with an IC₅₀ in

the 0.3 nM range.⁴ Microcystins are potent cyclic hepta- and pentapeptide toxins of the general structure $\text{cyclo}[\text{D-Ala-X-D-erythro-b-methyl-iso-Asp-Y-Adda-D-iso-Glu-N-methyldehydro-Ala}]$ where X and Y are variable L-amino acids.⁴ They are known to promote tumors *in vivo*, but, with the exception of hepatocytes, are impermeable to most cells *in vitro*.⁴

Table 1. Ser/Thr protein phosphatase classification.^{3,4}

Family	Subfamily	Characteristic
PSTPases	PP1	IC ₅₀ for okadaic acid 10-50 nM
	PP2A	IC ₅₀ for okadaic acid 0.5 nM
	PP2B (calcineurin)	Ca(II)-dependent; IC ₅₀ for okadaic acid >2000 nM
	PP2C	Mg(II)-dependent; not inhibited by okadaic acid
	PP3	IC ₅₀ for okadaic acid 4 nM

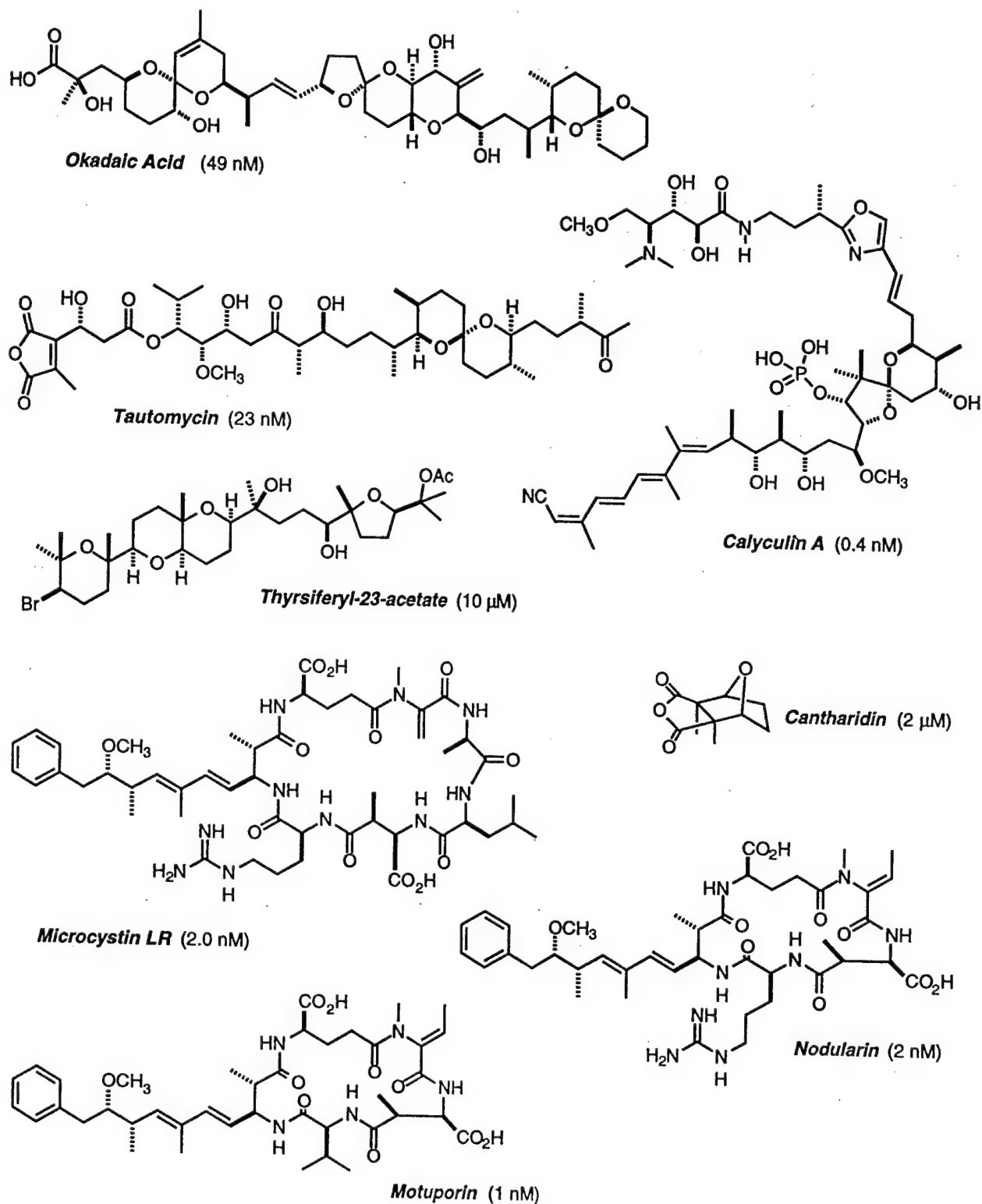
The large number of naturally occurring microcystins makes it possible to carry out a limited SAR study.¹⁵ Apparent IC₅₀'s for microcystins range between 0.05 to 5 nM, with similar preference for PP1, PP2A, and PP3 as found for okadaic acid and calyculin A.⁴ The substitution of alanine for arginine has little effect on phosphatase inhibitory potency; there is, however, a difference in relative cytotoxicity.¹⁵ The dehydroamino acid residue and the *N*-methyl substituents are also not critical. Crucial are the glutamic acid unit, since esterification leads to inactive compounds, and the overall shape of the Adda residue, since the (6*Z*)-isomer is inactive. Some variations in the Adda unit, specifically the *O*-demethyl and the *O*-demethyl-*O*-acetyl analogs, exert little effect on bioactivity, however. Considerable less information is available in the nodularin series, since fewer compounds are available; however, the general SAR appears similar to the microcystins.¹⁵ There are only slight differences in the inhibition profile; IC₅₀'s for PP1 and PP3 are 2 and 1 nM, respectively, which is about 50 times higher than the IC₅₀ for PP2A. The recently isolated motuporin (= [L-

Val²]nodularin) is even more potent with an IC₅₀ <1 nM for PP1.^{16,17} This secondary metabolite was isolated from a Papua New Guinea sponge and is the only member of the greater microcystin family that has thus far yielded to total synthesis.¹⁸

Tautomycin is produced by a terrestrial *Streptomyces* strain. This relatively unstable molecule inhibits PP1, PP2A and PP3 indiscriminately with an IC₅₀ in the 15 nM range.⁴ The remaining natural product inhibitors, thyransferyl-23-acetate and cantharidine, were shown to be somewhat selective, though weak (IC₅₀ 0.16-10 μ M) inhibitor of PP2A.^{19,20,21,22}

Despite some recent total synthesis efforts,²³ no SAR for calyculin A, tautomycin²⁴ or thyransferyl acetate were reported. High toxicity, especially hepatotoxicity, is commonly found with all natural PSTPase inhibitors, often limiting the range of feasible pharmacological studies, and appears to be intrinsically associated with a non-specific phosphatase inhibition.²⁵ Importantly, based on kinetic and competition binding studies, okadaic acid, calyculin A, tautomycin, and the microcystins appear to bind competitively at the same site of PSTPases.^{26,27,28,29} Since phosphatases are ubiquitous, precise tools in membrane and post-membrane signal transduction pathways, the development of selective inhibitors or activators of PSTPases that are cell-permeable, non-hepatotoxic, or broadly cytotoxic is of major significance for future progress in this field.

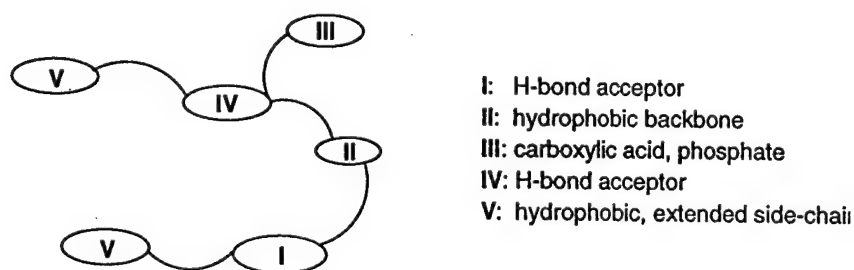
Figure 1. Natural product inhibitors of PSTPases (IC_{50} vs PP1).



Design and Synthesis of Calyculin A Analogs

The design of our PSTPase inhibitor library was based on the SAR available for the natural product inhibitors and assumed that the presence of a carboxylate, a nonpolar aromatic function, and hydrogen-bond acceptors and donors (e.g. a peptidomimetic group) in suitable spatial arrangements are sufficient for strong and selective binding. A pharmacophore model that addresses these criteria is shown in Figure 2. Traditional computational studies by Quinn et al. have identified a related structural model based on molecular modeling of okadaic acid, calyculin A, and microcystin LR.³⁰ Whereas computational studies of the minimal structural requirements for PSTPase inhibition aim for an accurate prediction of the important conformational and electronic features of the lead structures, our combinatorial^{31,32} analysis achieves this goal via a random optimization of the steric and electronic properties of the pharmacophore. Most marine natural products have evolved along an optimization of broad-range activity rather than specificity.³³ The structural variation present in a library of PSTPase inhibitors will allow the simultaneous exploration of high-affinity and high-specificity features providing selectivity beyond the natural product model.

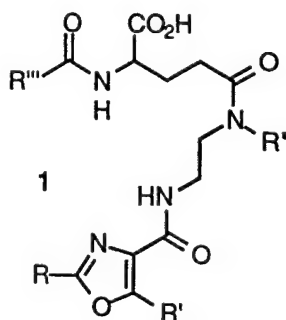
Figure 2. Pharmacophore model for PSTPase inhibitor library.



Specifically, we have designed compounds of structure **1** to provide a platform for functional group variation according to our pharmacophore model (Figure 3). The carboxylic acid moiety, crucial for bioactivity, is derived from glutamic acid. The substituent R attached

to the oxazole moiety of **1** can be varied within a broad range and should probably be mostly hydrophobic in nature. To a lesser extent, direct substitutions at the oxazole R' are possible that will explore the tolerance for bulky residues at this site. A variable and relatively flexible diamine segment serves as the spacer between oxazole moiety and carboxylic acid side chain in place of the synthetically less readily accessible spiroketal of calyculin A. A related *N*-methyl dehydroalanine residue is found in microcystin LR. The hydrophobicity of this subunit is modulated by *N*-alkylation with residues R". An acyl portion R'''CO is responsible for providing the molecule with a relatively rigid hydrophobic tail similar to the Adda amino acid side chain in microcystins and the tetraene cyanide in calyculin A.

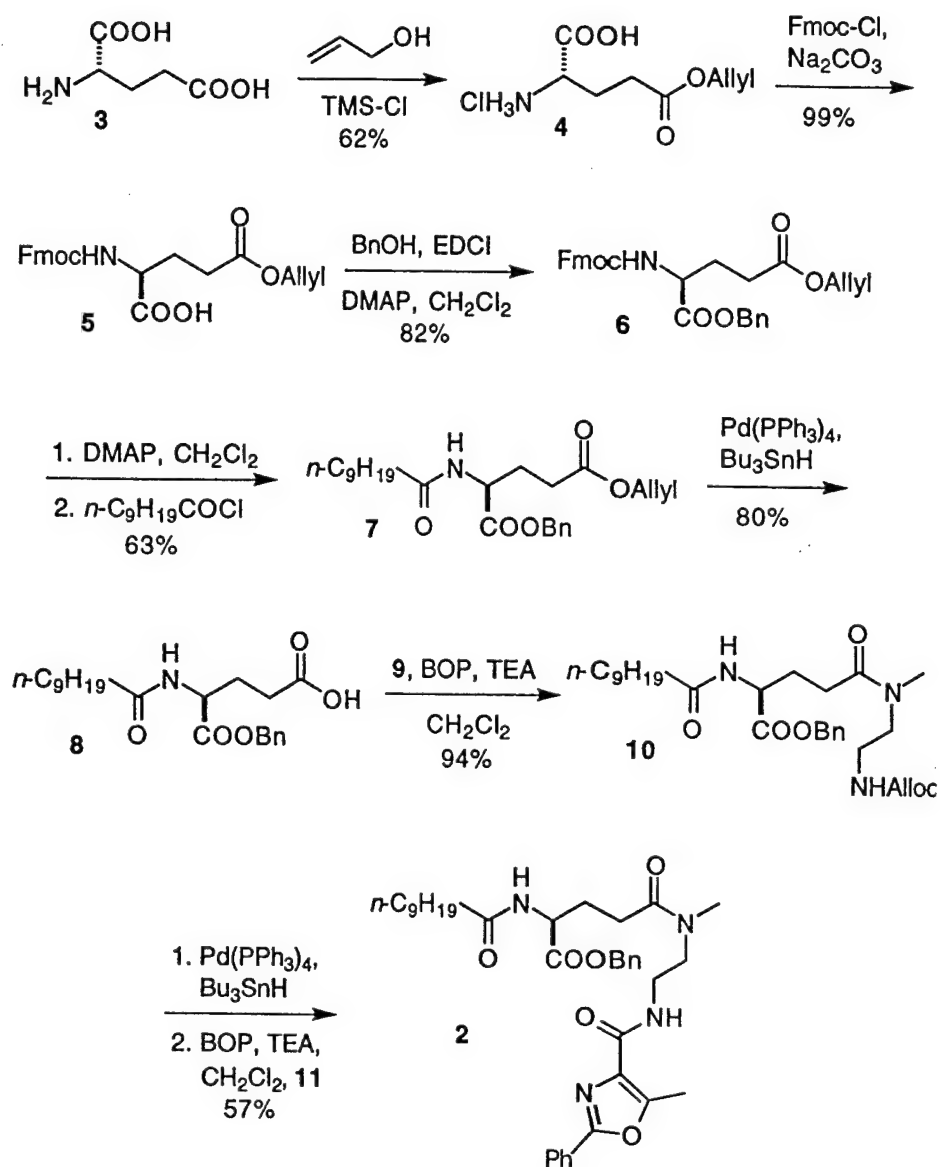
Figure 3. Parent structure for PSTPase inhibitor library synthesis.



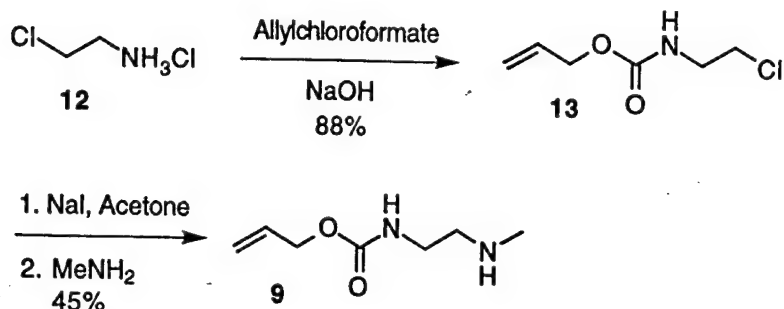
Initially, the development of an efficient approach for the combinatorial synthesis of target structures **1** focused on the optimization of the solution-phase synthesis of model compound **2** (Scheme 1). L-Glutamic acid **3** was protected in 62% yield as the γ -allyl ester using allyl alcohol and chlorotrimethylsilane.³⁴ Treatment with Fmoc-Cl followed by coupling to benzyl alcohol using 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDCI) provided the *tri*-protected amino acid **6** in 82% yield. The Fmoc protective group was subsequently removed by exposure to DMAP and the free amine was acylated *in situ* with decanoyl chloride to give amide **7** in 63% yield. Pd(0)-Catalyzed deprotection³⁵ of the allyl ester and coupling of the resulting acid **8** to ethylene diamine **9** in the presence of (1H-1,2,3-

benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP)³⁶ led to amide **10** in 75% yield. A versatile general route to monoprotected ethylene diamines was easily achieved by carbamoylation of 2-chloroethylamine monohydrochloride (**12**), Finkelstein reaction, and aminolysis (Scheme 2).

Scheme 1

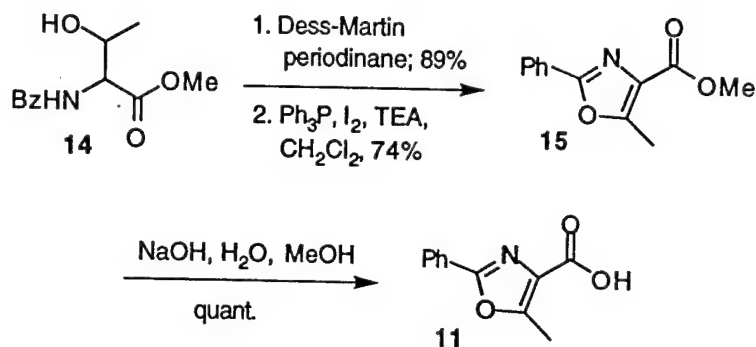


Scheme 2



Deprotection of the Alloc-group gave a primary amine which was coupled, *in situ*, to oxazole acid **11** using BOP as a coupling agent. The desired amide **2** was obtained in 57% yield for the two steps. The heterocyclic moiety **11** was efficiently prepared from *N*-benzoyl threonine (**14**) by side-chain oxidation and cyclodehydration with Dess-Martin reagent and electrophilic phosphorus, respectively,³⁷ followed by saponification of oxazole **15** (Scheme 3).

Scheme 3



The solution-phase preparation of calyculin analog **2** established the necessary general protocols for the preparation of a library of structural variants of the pharmacophore model **1** on solid support. We have successfully applied this basic strategy for the parallel

synthesis of 18 structural analogs (Scheme 4, Table 1). Coupling of diprotected glutamate **5** to the polystyrene-based Wang resin³⁸ with EDCI was performed on large scale and provided a supply of solid phase beads. The base-labile Fmoc protective group was removed by treatment with piperidine and THF, and the resin was distributed to 3 specially designed Schlenk filters equipped with suction adapters and inert gas inlets for maintaining steady bubbling. After the addition of solvent, hydrophobic residues $R'''\text{COCl}$ were added to each flask, which provided 3 different amide derivatives **17**. After filtration and rinsing of the resin, allyl esters **17** were deprotected via Pd(0) chemistry and each batch was distributed over 3 modified Schlenk filters, providing 9 different reaction sites for acylation. Addition of 3 different *N*-allyloxycarbonyl protected diamines in the presence of PyBroP³⁹ or CloP⁴⁰ as coupling agents extended the side chain carboxyl terminus of glutamic acid toward the desired heterocyclic moiety in **1**. The resulting 9 compounds **18** were each deprotected at the *N*-terminus and distributed over 2 additional Schlenk filters for the final segment condensation. Coupling with 2 different oxazole carboxylic acids in the presence of CloP and final purification by rinsing with solvent provided the phosphatase library **1** still attached to the solid support. Complete or partial cleavage with 50% trifluoroacetic acid was necessary to release the carboxylate which is required for biological activity. After filtration of the solid support and evaporation of the resulting mother liquor, the desired compounds **1** were obtained in a chemically pure and structurally well defined fashion ready for rapid throughput biological screening. In each case, the purity of the final compound was >60% according to spectroscopic analysis (¹H NMR, MS). The contamination was derived from incomplete couplings to the sterically hindered secondary amine moiety of Alloc-NHCH₂CH₂NH(R''). A small sample of resin had been routinely cleaved for reaction monitoring, but this coupling was difficult to drive to completion. Mass recovery was essentially quantitative. We are still in the process of further optimizing the reaction sequence and are confident that purities of >80% for the final material **1** can routinely be achieved after improvement of the coupling step.

Scheme 4

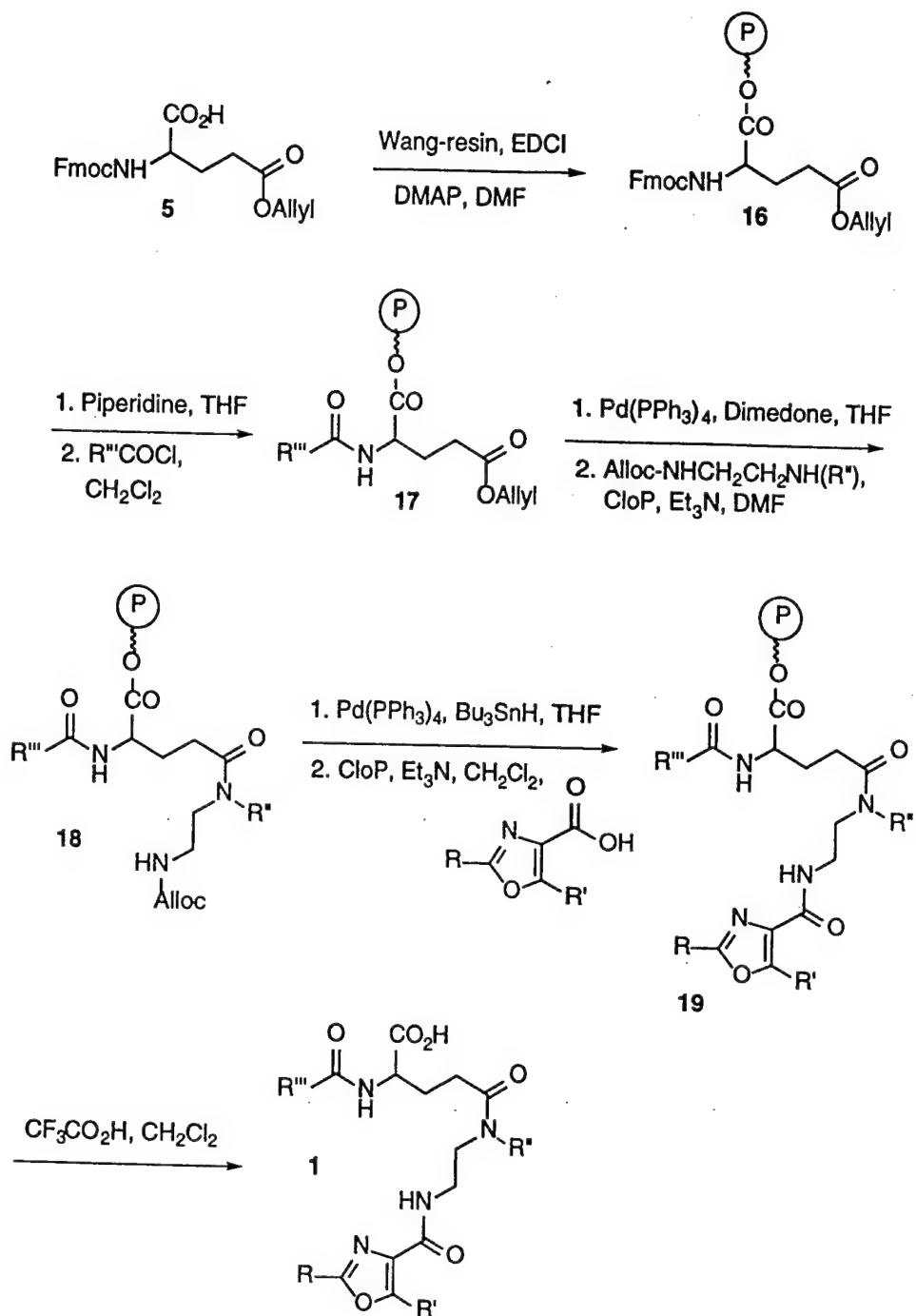


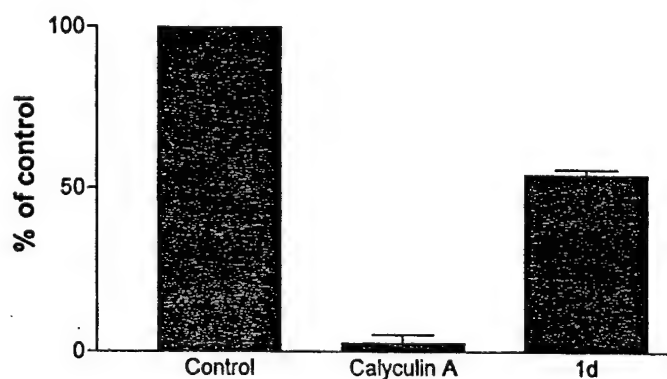
Table 1. Test library of 18 structural variants of pharmacophore model **1** prepared according to Scheme 4.

Compound	R	R'	R''	R'''
1a	Ph	CH ₃	CH ₃	<i>n</i> -C ₉ H ₁₉
1b	Ph	CH ₃	<i>n</i> -C ₆ H ₁₃	<i>n</i> -C ₉ H ₁₉
1c	Ph	CH ₃	Bn	<i>n</i> -C ₉ H ₁₉
1d	Ph	Ph	CH ₃	<i>n</i> -C ₉ H ₁₉
1e	Ph	Ph	<i>n</i> -C ₆ H ₁₃	<i>n</i> -C ₉ H ₁₉
1f	Ph	Ph	Bn	<i>n</i> -C ₉ H ₁₉
1g	Ph	CH ₃	CH ₃	PhCH ₂ CH ₂
1h	Ph	CH ₃	<i>n</i> -C ₆ H ₁₃	PhCH ₂ CH ₂
1i	Ph	CH ₃	Bn	PhCH ₂ CH ₂
1j	Ph	Ph	CH ₃	PhCH ₂ CH ₂
1k	Ph	Ph	<i>n</i> -C ₆ H ₁₃	PhCH ₂ CH ₂
1l	Ph	Ph	Bn	PhCH ₂ CH ₂
1m	Ph	CH ₃	CH ₃	PhCH=CH
1n	Ph	CH ₃	<i>n</i> -C ₆ H ₁₃	PhCH=CH
1o	Ph	CH ₃	Bn	PhCH=CH
1p	Ph	Ph	CH ₃	PhCH=CH
1q	Ph	Ph	<i>n</i> -C ₆ H ₁₃	PhCH=CH
1r	Ph	Ph	Bn	PhCH=CH

Preliminary Biochemical and Biological Analysis of Library 1

We have begun to evaluate the ability of compounds **1a-r** to inhibit PP1 and PP2A. Initial studies were conducted in collaboration with Drs. A. Boynton and D. Messner,⁴¹ with their previously described assay.^{25,29} These preliminary studies demonstrated that several members of our library inhibit protein phosphatases PP1 or PP2A by >50% at concentrations of 100 μ M. We have further examined the ability of one member of the library to inhibit the catalytic activity of PP2A. As demonstrated in Figure 4, calyculin A inhibited PP2A activity at 10 nM, and compound **1d** caused 50% inhibition at 100 μ M. These results document that our minimal structure retained the ability to inhibit the catalytic activity of Ser/Thr phosphatase. More comprehensive analyses are currently being conducted.

Figure 4. Inhibition of PP2A activity by compound **1d**. The catalytic subunit of PP2A was incubated with vehicle alone (control), calyculin A (10 nM), or **1d** (100 μ M), and the dephosphorylation of the substrate fluorescein diphosphate determined spectrofluorometrically. Mean results to two independent experiments are shown; bars indicate the range.



PSTPases are intracellular targets and, thus, we have examined the antiproliferative effects of members of the library to indirectly assess whether our compounds might enter cells. Exponentially growing human MDA-MB-231 breast carcinoma cells were exposed to all compounds at the highest available concentrations, which ranged from 30 to 100 μM . With the exception of two compounds, all lacked significant growth inhibitory activity. Compound **1h** caused 50% growth inhibition at 20 μM but had no further cytotoxicity at higher drug concentrations. Compound **1f** caused 50% growth inhibition at 100 μM and had a clear concentration-dependency (Figure 5). Cell proliferation is coordinated by phosphorylation of cyclin-dependent kinases and tightly regulated by both kinases and phosphatases.⁴² Thus, inhibition of Ser/Thr phosphatases such as PP2A or PP1 can result in disrupted cell cycle transition with restriction at discrete points in the cell cycle. Exponentially growing human MDA-MB-231 breast cancer cell populations (population doubling time of approximately 30-35 h) typically have approximately 50% of all cells in the S or DNA synthetic phase of the cell cycle (Figure 6, A and C). In contrast, when MDA-MB-231 cells were incubated for 48 h with 100 μM compound **1f**, there was prominent accumulation in the G1 phase with a concomitant decrease in both S and G2/M phases (Figure 6, B and C). Incubation of MDA-MB-231 cells for 72 h with 88 μM **1f** also caused a prominent accumulation in the G1 phase (Figure 6, D).

Figure 5. Antiproliferative effect of compound **1f** against human MDA-MB-231 breast cancer cells.

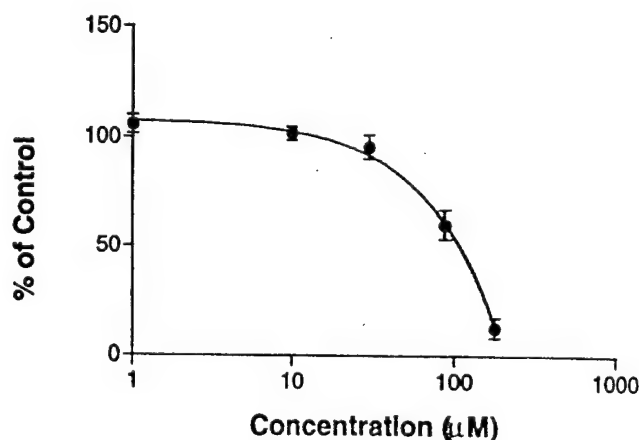
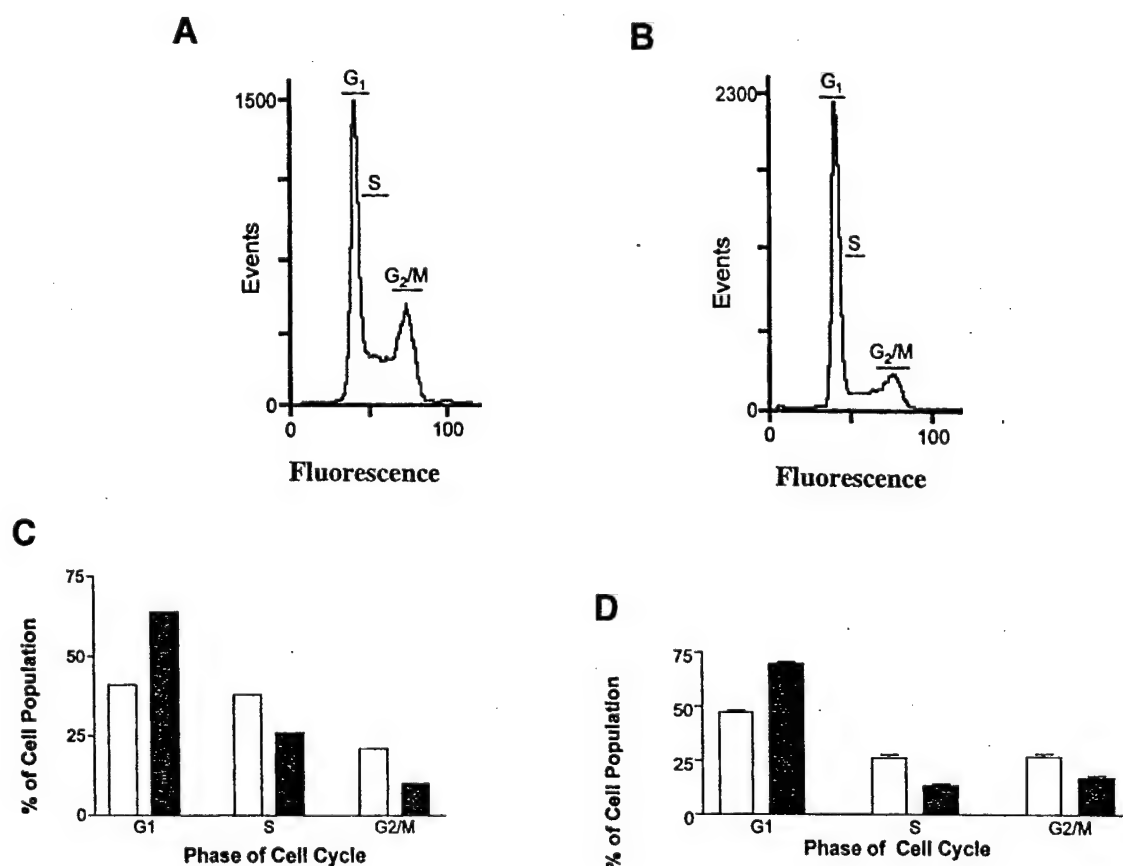


Figure 6. Cell cycle distribution of human breast cancer cells after treatment with compound **1f** determined by flow cytometry. Panel A. Flow cytometry analysis of MDA-MB-231 cells treated with vehicle alone. Panel B. Flow cytometry analysis 48 h after treatment with 100 μ M compound **1f**. Fluorescence channel measures intracellular propidium iodide concentration, an index of DNA content. Horizontal bars are the gating positions that allow for cell cycle analysis. Panel C. MDA-MB-231 cell cycle distribution 48 h after continuous treatment with 100 μ M compound **1f**. This is the result of one experiment. Open bars are control cells and black bars are cells treated with **1f**. Panel D. Cell cycle distribution 72 h after continuous treatment with 88 μ M **1f**. The mean values were obtained from 3 independent determinations. Open bars are control cells and black bars are cells treated with 88 μ M **1f**. The SE of the mean are displayed.



Discussion

Due to the limited character of previous SAR studies of the available natural product serine/threonine phosphatase inhibitors, the design of a small-molecule pharmacophore model has to allow for considerable structural variation. The combinatorial chemistry strategy is therefore ideally suited to address this problem. Among the characteristic structural features of calyculin A and the microcystins, the presence of a carboxylate, amide, oxazole, and lipophilic moieties are important features shared with our first generation lead structure **1**. The use of traditional amide coupling protocols combined with transition metal susceptible protective groups provided the basis for the parallel synthesis of 18 analogs of **1** via a solid-phase chemistry. We have begun to test library both for biochemical and biological activity. Figure 4 demonstrates that the basic pharmacophore that we have identified retains the ability to inhibit Ser/Thr phosphatases. We have not yet evaluated other members of our library with this assay, but inhibition of PP2A and PP1 has been established in preliminary studies for several members of our library. Compound **1a-r** were further subjected to an assay for cytotoxicity and apoptosis in human breast carcinoma cells, and two members (**1h** and **1f**) with an IC_{50} of $<100 \mu M$ were found.⁴³ Interestingly, **1d**, which can block PP2A activity did not appear to be cytotoxic. This lack of biological activity may be due to poor cell penetration of cellular metabolism. Compound **1h** did not suppress cell proliferation significantly more than 50% in our assay and thus was not examined further. Compound **1f**, however, exhibited a concentration-dependent inhibition in proliferation of MDA-MB-231 cells and flow cytometry data confirmed blockage in cell cycle progression at the G1 checkpoint. Although PSTPase inhibitors such as okadaic acid and calyculin A often are found to block cells in G2/M, a concentration-dependent cell cycle arrest at the G1/S interface similar to that seen by us has been detected with some cells.⁴⁴ An additional attractive target phosphatase that might control the G1/S transition would be the dual specificity phosphatase cdc25A.⁴⁵

Studies of the effect of **1a-r** on cdc25A and other phosphatases that may control cell cycle checkpoints are currently in progress.

These results clearly demonstrate the feasibility of using a combinatorial approach based on a natural product lead to identify novel antiproliferative and potential antineoplastic agents. Since cellular signal transduction is regulated by reversible enzymatic phosphorylation of serine, threonine and tyrosine residues on proteins, we expect that appropriately substituted, phosphatase-specific isomers of **1** will become important probes for transcription factor regulation, cell cycle control, and membrane and post-membrane signaling pathways. We are actively pursuing the synthesis and rapid-screening assays of much larger libraries based on **1** to identify more potent and more specific analogs.

Experimental Section

General Methods. All glassware was dried in an oven at 150 °C prior to use. THF and dioxane were dried by distillation over Na/benzophenone under a nitrogen atmosphere. Dry CH₂Cl₂, DMF and CH₃CN were obtained by distillation from CaH₂.

2-Amino-pentanedioic acid 5-allyl ester (4). To a stirred suspension of 2.5 g (16.9 mmol) of L-glutamic acid (**3**) in 40 mL of dry allyl alcohol was added dropwise 5.4 mL (42.3 mmol) of chlorotrimethylsilane. The suspension was stirred at 22 °C for 18 h and poured into 300 mL of Et₂O. The resulting white solid was filtered off, washed with Et₂O, and dried in vacuo to provide 3.80 g (62%) of **4**: Mp 133-134.5 °C (Et₂O); IR (KBr) 3152, 2972, 2557, 1738, 1607, 1489, 1450, 1289, 1366, 1264, 1223, 1177, 1146, 121, 1084 cm⁻¹; ¹H NMR (D₂O) δ 5.8-5.7 (m, 1 H), 5.14 (dd, 1 H, *J* = 1.4, 17.3 Hz), 5.09 (dd, 1 H, *J* = 1.0, 10.4 Hz), 4.44 (d, 2 H, *J* =

5.6 Hz), 3.92 (t, 1 H, $J = 6.8$ Hz), 2.48 (t, 2 H, $J = 7.0$ Hz), 2.1-2.0 (m, 2 H); ^{13}C NMR (DMSO- d_6) δ 171.5, 170.6, 132.7, 117.9, 64.7, 51.2, 29.3, 25.2; MS (EI) m/z (relative intensity) 188 (63), 142 (72), 128 (27), 100 (21), 85 (100), 74 (32), 56 (73).

2-(9-H-Fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester (5). To 20 mL of dioxane was added 1.5 g (6.7 mmol) of ester 4. The resulting suspension was treated with 16.8 mmol (17.7 mL of a 10% solution) of sodium carbonate at 0 °C, stirred for 5 min and treated with 1.74 g (6.7 mmol) of Fmoc-Cl dissolved in 10 mL of dioxane. The reaction mixture was warmed to 22 °C, stirred for 3 h, poured into 50 mL of H_2O and extracted with Et_2O (2X 25 mL). The aqueous layer was cooled to 0 °C, acidified to pH 1 with concentrated HCl, and extracted with EtOAc (3X25 mL). The resulting organic layer was dried (Na_2SO_4) and concentrated in vacuo to give 2.72 g (99%) of 5 as a viscous oil: $[\alpha]_D +8.5^\circ$ (c 2.8, CHCl_3 , 21 °C); IR (neat) 3312, 3061, 2951, 2361, 2349, 2332, 1725, 1528, 1447, 1414, 1325, 1254, 1117, 1078, 1049 cm^{-1} ; ^1H NMR δ 11.09 (bs, 1 H), 7.73 (d, 2 H, $J = 7.5$ Hz), 7.57 (d, 2 H, $J = 5.1$ Hz), 7.4-7.25 (m, 4 H), 6.0-5.85 (m, 1 H), 5.76 (d, 1 H, $J = 8.1$ Hz), 5.30 (d, 1 H, $J = 19.5$ Hz), 5.21 (d, 1 H, $J = 10.5$ Hz), 4.6-4.35 (m, 5 H), 4.19 (t, 1 H, $J = 6.6$ Hz), 2.5-2.2 (m, 4 H); ^{13}C NMR δ 175.6, 172.6, 156.2, 143.7, 143.5, 141.2, 131.7, 127.6, 127.0, 125.0, 119.9, 118.4, 67.1, 65.4, 53.1, 46.9, 30.2, 27.1; MS (EI) m/e (relative intensity) 409 (7), 351 (19), 338 (12), 280 (11), 239 (11), 196 (12), 178 (100), 165 (40); HRMS (EI) calculated for $\text{C}_{23}\text{H}_{23}\text{NO}_6$: 409.1525, found: 409.1501.

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester 1-benzyl ester (6). To a solution of 1.5 g (36.6 mmol) of 5 in 5 mL of CH_2Cl_2 was added 0.42 mL (40.3 mmol) of benzyl alcohol, 0.912 g (47.6 mmol) of EDCI, and 45 mg (3.66 mmol) of dimethylaminopyridine (DMAP). The reaction mixture was stirred at 22 °C for 6 h, diluted with 20 mL of CH_2Cl_2 , and extracted with H_2O (1x15 mL), 0.1 M HCl (2x15 mL), and brine (2x10 mL). The organic layer was dried (Na_2SO_4), concentrated in vacuo, and

chromatographed on SiO₂ (Hexanes/EtOAc, 5:1) to give 1.83 g (82%) of **6** as a white solid: Mp 66.2-67.1 °C (EtOAc/Hexanes); [α]_D +1.4° (*c* 1.64, CHCl₃, 21 °C); IR (neat) 3314, 1726, 1682, 1527, 1443, 1414, 1383, 1254, 1173, 1099, 1082, 980, 754, 735 cm⁻¹; ¹H NMR δ 7.75 (d, 2 H, *J* = 7.4 Hz), 7.59 (d, 2 H, *J* = 7.1 Hz), 7.41-7.27 (m, 9 H), 5.95-5.85 (m, 1 H), 5.44 (d, 1 H, *J* = 8.2 Hz), 5.34-5.19 (m, 4 H), 4.56 (d, 2 H, *J* = 5.6 Hz), 4.5-4.4 (m, 3 H), 4.21 (t, 1 H, *J* = 7.0 Hz), 2.5-2.0 (m, 4 H); ¹³C NMR δ 172.2, 171.6, 155.8, 143.7, 143.5, 141.1, 135.0, 131.8, 128.5, 128.3, 128.1, 127.6, 126.9, 124.9, 119.8, 118.3, 67.2, 66.9, 66.2, 53.3, 47.0, 28.0, 27.3; MS (FAB, MNBA/MeOH) *m/z* (relative intensity) 500 ([M+H]⁺, 40), 465 (8), 448 (14), 433 (12), 413 (8), 386 (38), 371 (24), 349 (9), 324 (16), 309 (26), 293 (11), 265 (10), 247 (24), 231 (56), 215 (39), 202 (26), 191 (24), 179 (67), 165 (48), 154 (67), 143 (31), 133 (71), 117 (100).

2-Decanoylamino-pentanedioic acid 5-allyl ester 1-benzyl ester (7). To a suspension of 1 g (2.0 mmol) of **6** in 10 mL of CH₂Cl₂ was added 1 g (8.2 mmol) of DMAP. The reaction mixture was stirred at 22 °C for 24 h, treated with 0.62 mL (3.0 mmol) of decanoyl chloride, stirred for 2 h at 22 °C, and extracted with saturated sodium bicarbonate solution (2x10 mL). The organic layer was dried (Na₂SO₄), evaporated to dryness, and the residue was chromatographed on SiO₂ (Hexanes/EtOAc, 5:1) to give 548 mg (63%) of **7** as a viscous oil: IR (neat) 3293, 3063, 2924, 2855, 1740, 1649, 1534, 1453, 1379, 1175, 986, 930 cm⁻¹; ¹H NMR δ 7.26 (s, 5 H), 6.68 (d, 1 H, *J* = 7.8 Hz), 5.85-5.75 (m, 1 H), 5.22 (d, 1 H, *J* = 17.3 Hz), 5.14 (d, 1 H, *J* = 10.4 Hz), 5.08 (s, 2 H), 4.63-4.57 (m, 1 H), 4.48 (d, 2 H, *J* = 5.6 Hz), 2.38-2.28 (m, 2 H), 2.2-2.1 (m, 3 H), 2.0-1.9 (m, 1 H), 1.55 (t, 2 H, *J* = 6.9 Hz), 1.20 (bs, 12 H), 0.82 (t, 3 H, *J* = 5.9 Hz); ¹³C NMR δ 173.0, 172.1, 171.6, 135.0, 131.7, 128.2, 128.1, 127.8, 117.9, 66.8, 64.9, 51.3, 36.0, 31.6, 29.9, 29.1, 29.0, 26.8, 25.3, 22.3, 13.8; MS (EI) *m/z* (relative intensity) 431 (12), 319 (21), 296 (51), 142 (100), 124 (31), 91 (91); HRMS (EI) *m/z* calculated for C₂₅H₃₇NO₅: 431.2672, found: 431.2673.

2-Decanoylamino-pentanedioic acid 1-benzyl ester (8). To a solution of 752 mg (1.74 mmol) of 2-decanoylamino-pentanedioic acid **7** in 10 mL of CH_2Cl_2 was added 100 mg (0.087 mmol) of tetrakis(triphenylphosphine) $\text{Pd}(0)$ followed by 0.52 mL (1.9 mmol) of tributyltin hydride. After 15 min, the reaction mixture was quenched with 10 mL of a 10% HCl solution. The aqueous layer was reextracted with 15 mL of CH_2Cl_2 and the organic layer dried (Na_2SO_4), concentrated in vacuo, and chromatographed on SiO_2 (Hexanes/EtOAc, 9:1) to provide 545 mg (79.9%) of **8** as a thick oil: $[\alpha]_D +2.8^\circ$ (*c* 1.2, CHCl_3 , 21 °C); IR (neat) 3351, 3064, 2995, 2852, 1738, 1712, 1657, 1536, 1454, 1380, 1364, 1265, 1209, 1183, 1121, 739 cm^{-1} ; ^1H NMR δ 10.9-10.7 (bs, 1 H), 7.22 (s, 5 H), 6.58 (d, 1 H, $J = 7.8$ Hz), 5.09 (s, 2 H), 4.63 (dd, 1 H, $J = 8.1, 12.9$ Hz), 2.4-2.25 (m, 2 H), 2.2-2.1 (m, 3 H), 2.0-1.9 (m, 1 H), (m, 6 H), 1.53 (t, 2 H, $J = 6.6$ Hz), 1.19 (bs, 12 H), 0.81 (t, 3 H, $J = 6.0$ Hz); ^{13}C NMR δ 176.9, 174.0, 171.8, 134.9, 128.5, 128.4, 128.1, 67.3, 51.4, 36.2, 31.7, 29.9, 29.3, 29.2, 29.1, 27.0, 25.5, 22.5, 14.0; MS (EI) m/z (relative intensity) 391 (54), 373 (62), 279 (13), 256 (19), 178 (27), 178 (23), 155 (13), 146 (6), 130 (7), 102 (100); HRMS (EI) m/z calculated for $\text{C}_{22}\text{H}_{33}\text{NO}_5$: 391.2358, found: 391.2350.

4-[(2-Allyloxycarbonylamino-ethyl)-methyl-carbamoyl]-2-decanoylamino-butyric acid benzyl ester (10). To a solution of 526 mg (1.3 mmol) of **8** in 10 mL of CH_2Cl_2 was added 225 μL (1.61 mmol) of triethylamine and 320 mg (2.0 mmol) of secondary amine **9**. The solution was stirred at 22 °C for 5 min, treated with 710 mg (1.61 mmol) of benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent), stirred at 22 °C for 10 min, concentrated in vacuo, dissolved in 15 mL of EtOAc, and extracted with 2 M HCl solution. The organic layer was chromatographed on SiO_2 (Hexanes/EtOAc, 1:3) to give 715 mg (94%) of **10** as a clear oil: $[\alpha]_D +5.3$ (*c* 0.58, CHCl_3 , 21 °C); IR (neat) 3420, 3250, 2924, 1713, 1680, 1657, 1642, 1632, 1537, 1495, 1470, 1455, 1252, 845 cm^{-1} ; ^1H NMR δ 7.35-7.2 (bs, 5 H), 6.97 (d, 0.3 H, $J = 7.5$ Hz), 6.82 (d, 0.7 H, $J = 7.3$ Hz), 5.9-5.6 (m, 2 H), 5.3-5.1 (m, 4 H), 4.65-4.5 (m, 1 H), 4.50 (d, 2 H, $J = 4.9$ Hz); 3.55 (t, 1 H, $J = 7.0$ Hz), 3.35-3.1 (m, 3

H), 2.85 (s, 3 H), 2.4-1.8 (m, 6 H), 1.65-1.5 (m, 2 H), 1.22 (bs, 12 H), 0.84 (t, 3 H, $J = 6.1$ Hz); ^{13}C NMR (MeOD) δ 176.4, 176.3, 174.4, 174.2, 173.2, 158.6, 137.1, 134.3, 134.2, 132.9, 129.5, 129.2, 129.1, 117.6, 117.4, 67.8, 66.3, 66.2, 53.5, 53.3, 39.6, 39.3, 36.7, 36.6, 34.2, 32.9, 30.5, 30.4, 30.3, 30.2, 29.7, 27.6, 26.8, 23.6, 14.5; MS (EI) m/z (relative intensity) 531 (16), 473 (37), 418 (16), 396 (26), 374 (38), 361 (17), 338 (87), 220 (54), 184 (52), 155 (36), 130 (29), 101 (37), 91 (100); HRMS (EI) m/z calculated for $\text{C}_{29}\text{H}_{45}\text{N}_3\text{O}_6$: 531.3308, found: 531.3316.

2-Decanoylamino-4-(methyl-{3-[5-methyl-2-phenyl-oxazole-4-carbonyl]-ethyl}-carbamoyl)-butyric acid benzyl ester (2). To a solution of 193 mg (0.363 mmol) of **10** in 15 mL of CH_2Cl_2 was added 20 mg (0.018 mmol) of tetrakis(triphenylphosphine) $\text{Pd}(0)$, 127 μL (0.472 mmol) of tributyltin hydride, and 20 μL of H_2O . The reaction mixture was stirred at 22 $^\circ\text{C}$ for 5 min, filtered through a plug of basic Al_2O_3 and treated with 150 mg (0.726 mmol) of oxazole **11**, 60 μL (0.436 mmol) of triethylamine, and 192 mg (0.436 mmol) of BOP reagent. The reaction mixture was stirred for 30 min at 22 $^\circ\text{C}$, diluted with 10 mL of CH_2Cl_2 , and extracted with saturated NaHCO_3 solution, 1M HCl, and brine. The organic layer was concentrated in vacuo and chromatographed on SiO_2 (Hexanes/EtOAc, 1:1) to give 131 mg (57%) of **2** as a viscous oil: $[\alpha]_D -0.8^\circ$ (c 1.32, CHCl_3 , 21 $^\circ\text{C}$); IR (neat) 3476, 3415, 3311, 3065, 2925, 2854, 1741, 1649, 1526, 1491, 1379, 1338, 1264, 1240, 1200, 1174, 1070, 711 cm^{-1} ; ^1H NMR 8.0-7.95 (m, 2 H), 7.5-7.4 (m, 2 H), 7.33 (bs, 6 H), 6.93 (d, 0.3 H, $J = 7.0$ Hz), 6.85 (d, 0.7 H, $J = 7.2$ Hz), 5.18-5.07 (m, 2 H), 4.65-4.55 (m, 1 H), 3.7-3.3 (m, 4 H), 2.98 (s, 1 H), 2.96 (s, 2 H), 2.71 (d, 3 H, $J = 2.6$ Hz), 2.6-2.0 (m, 6 H), 1.58 (t, 2 H, $J = 6.8$ Hz), 1.3-1.1 (bs, 12 H), 0.86 (t, 3 H, $J = 6.9$ Hz); ^{13}C NMR δ 173.3, 172.8, 172.0, 171.9, 182.5, 158.6, 153.2, 152.8, 135.9, 130.7, 130.6, 129.7, 128.8, 128.5, 128.3, 128.2, 126.7, 126.5, 126.2, 66.9, 52.2, 52.1, 48.9, 47.6, 37.2, 37.1, 36.4, 36.3, 36.2, 34.1, 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 28.9, 26.8, 26.6, 25.5, 22.8, 14.1, 11.8; MS (EI) m/z (relative intensity) 632 (38), 497

(9), 405 (18), 374 (22), 260 (21), 220 (42), 186 (56), 105 (18), 91 (100); HRMS calculated for $C_{36}H_{48}N_4O_6$: 632.3574, found: 632.3572.

(2-Chloro-ethyl)-carbamic acid allyl ester (13). A solution of 2.5 g (22 mmol) of chloroethylamine hydrochloride in 10 mL of 6 M NaOH was cooled to 0 °C and treated dropwise with 2.7 mL (25.9 mmol) of allyl chloroformate while keeping the pH at 9 by addition of 6 M NaOH solution. The reaction was then warmed to 22 °C, stirred for 2 h, and extracted with THF. The organic layer was dried (Na_2SO_4), concentrated in vacuo, and chromatographed on SiO_2 (Hexanes/EtOAc, 9:1) to give 3.1 g (88%) of **13** as a yellow oil: IR (neat) 3333, 2949, 2348, 1705, 1647, 1529, 1433, 1368, 1248, 1190, 1144, 1061, 991, 929, 776 cm^{-1} ; 1H NMR δ 6.05-5.85 (m, 1 H), 5.55-5.35 (bs, 1 H), 5.26 (dd, 1 H, J = 1.5, 17.1 Hz), 5.18 (dd, 1 H, J = 1.0, 10.4), 4.54 (d, 2 H, J = 5.5 Hz), 3.57 (t, 2 H, J = 5.5 Hz), 3.5-3.35 (m, 2 H); ^{13}C NMR δ 156.0, 132.5, 117.7, 65.6, 43.8, 42.7.

(2-Methylamino-ethyl)-carbamic acid allyl ester (9). A solution of 14 g (86 mmol) of **13** and 25 g (172 mmol) of sodium iodide in 40 mL of acetone was refluxed for 18 h, concentrated in vacuo, dissolved in H_2O , and extracted with CH_2Cl_2 . The organic layer was dried (Na_2SO_4) and cooled to 0° C. Methyl amine was bubbled through the reaction mixture until the solution was saturated. The reaction mixture was warmed to 22 °C, stirred for 36 h, concentrated in vacuo and chromatographed on SiO_2 (EtOAc) to produce 6.14 g (45%) of **9** as a yellow oil: IR (neat) 3306, 2938, 2313, 1844, 1703, 1651, 1525, 1460, 1383, 1256, 1144, 995, 927, 775 cm^{-1} ; 1H NMR δ 5.95-5.8 (m, 1 H), 5.28 (dd, 1 H, J = 1.4, 17.3 Hz), 5.18 (d, 1 H, J = 10.4 Hz), 4.54 (d, 2 H, J = 5.3 Hz), 4.9-4.6 (bs, 1 H), 3.34 (q, 2 H, J = 5.6 Hz), 2.79 (t, 2 H, J = 5.6 Hz), 2.47 (s, 3 H); ^{13}C NMR δ 157.22, 132.8, 117.6, 65.5, 50.7, 39.7, 35.4; MS (EI) m/e (relative intensity) 158 (32), 138 (17), 129 (25), 101 (13), 84 (12), 73 (13), 57 (100).

5-Methyl-2-phenyl-oxazole-4-carboxylic acid methyl ester (15). A solution of 750 mg (3.2 mmol) of **14** in 10 mL of CH_2Cl_2 was treated with 1.61 g (3.8 mmol) of Dess-Martin reagent. The reaction was stirred at 22 °C for 10 min, concentrated in vacuo, and chromatographed on SiO_2 (Hexanes/EtOAc, 3:2) to give 658 mg (89%) of 2-benzoylamino-3-oxo-butyric acid methyl ester. Alternatively, a solution of 9.12 g (38 mmol) of **14** in 80 mL of CH_2Cl_2 was cooled to -23 °C and treated with 16.1 mL (115 mmol) of triethylamine and a solution of 18.3 g (115 mmol) of SO_3 -pyridine complex in 60 mL of dry DMSO. The reaction mixture was warmed to 22 °C, stirred for 30 min, then cooled to -48 °C and quenched with 20 mL of saturated NaHCO_3 . The solution was extracted with 50 mL of Hexanes/EtOAc, 2:1. The aqueous layer was reextracted with Hexanes/ Et_2O , 2:1, and the combined organic layers were washed with brine, dried (Na_2SO_4), and chromatographed (Hexanes/EtOAc, 3:2) to give 7.1 g (79%) of 2-benzoylamino-3-oxo-butyric acid methyl ester as a white solid: Mp 112.7-113.3 °C (Hexanes/EtOAc); IR (neat) 3402, 1734, 1662, 1599, 1578, 1510, 1478, 1435, 1354, 1269, 1156, 1121, 912, 804, 714 cm^{-1} ; ^1H NMR 8.2-8.1 (bs, 1 H), 8.0-7.4 (m, 5 H), 5.49 (s, 1 H), 3.86 (s, 3 H), 2.33 (s, 3 H); ^{13}C NMR δ 168.2, 167.2, 132.6, 132.5, 132.1, 128.7, 127.3, 83.9, 54.2, 23.2; MS (EI) m/e (relative intensity) 235 (13), 208 (18), 192 (8), 121 (7), 105 (100), 77 (58).

A solution of 277 mg (1.06 mmol) of triphenylphosphine, 268 mg (1.06 mmol) of iodine, and 0.29 mL (2.11 mmol) of triethylamine in 5 mL of CH_2Cl_2 was cooled to -48 °C and treated with a solution of 124 mg (0.528 mmol) of 2-benzoylamino-3-oxo-butyric acid methyl ester in 5 mL of CH_2Cl_2 . The reaction mixture was warmed to 22 °C, stirred for 20 min, transferred to a separatory funnel and extracted with aqueous sodium thiosulfate followed by saturated sodium bicarbonate. The organic layer was concentrated in vacuo and chromatographed on SiO_2 (Hexanes/EtOAc, 9:1) to give 84.4 mg (74%) of **15** as a white solid: Mp 89.3-89.9 °C (Hexanes/EtOAc); IR (neat) 3025, 1717, 1610, 1561, 1485, 1436, 1348, 1323, 1302, 1285, 1235, 1188, 1103, 1072, 1057, 1022 cm^{-1} ; ^1H NMR 8.1-7.95 (m, 2 H), 7.5-7.3 (m, 3 H), 3.92 (s, 3 H), 2.68 (s, 3 H); ^{13}C NMR δ 162.7, 159.5, 156.3, 130.8, 128.8, 128.6, 128.3, 126.4,

51.9, 11.98; MS (EI) m/z (relative intensity) 231 (6), 217 (51), 185 (55), 105 (100), 77 (41), 44 (64); HRMS (EI) m/z calculated for $C_{12}H_{11}NO_3$: 217.0739, found: 217.0729.

5-Methyl-2-phenyl-oxazole-4-carboxylic acid (11). A solution of 2.07 g (9.5 mmol) of **15** in 20 mL of 3 M NaOH and 12 mL of MeOH was stirred at 22 °C for 2 h and extracted with Et_2O . The aqueous layer was acidified to pH 1 with concentrated HCl and extracted with EtOAc. The organic layer was dried (Na_2SO_4), and concentrated in vacuo to give 1.84 g (95%) of **11** as an off-white solid: Mp 182.3-182.6 °C (EtOAc/Hexanes); IR (neat) 3200, 2950, 2932, 2890, 2363, 2336, 1694, 1682, 1611, 1563, 1483, 1450, 1337, 1255, 1192, 1117, 1053, 1020 cm^{-1} ; 1H NMR δ 10.2-9.9 (bs, 1 H), 8.2-7.9 (m, 2 H), 7.6-7.4 (m, 3 H), 2.75 (s, 3 H); ^{13}C NMR (CD_3OD) δ 164.6, 160.7, 157.4, 131.9, 129.8, 129.6, 127.3, 127.2, 12.1; MS (EI) m/z (relative intensity) 203 (53), 185 (24), 157 (13), 116 (17), 105 (100), 89 (21), 77 (33), 63 (16); HRMS calculated for $C_{11}H_9NO_3$: 203.0582, found: 203.0583.

Solid Phase Chemistry:

Step 1, 5 \rightarrow 16. In a medium porosity Schlenk filter apparatus was placed 750 mg of Wang resin (0.96 mmol/g, 0.72 mmol of active sites). The resin was suspended in 12 mL of dry DMF and a stream of nitrogen was forced up through the filter at a rate which allowed the solvent to gently bubble. To this reaction mixture was added 1.47 g (3.6 mmol) of **5**. The suspension was agitated for 5 min and treated with 26 mg (0.216 mmol) of DMAP and 550 mg (2.88 mmol) of EDCI, agitated at 22 °C for 18 h and filtered, and the resin was washed with DMF (2x10 mL), H_2O (3x10 mL), THF (3x10 mL), and CH_2Cl_2 (3x10 mL). The resin was dried under vacuum and the remaining active sites were capped by addition of 10 mL of CH_2Cl_2 and 10 mL of acetic anhydride along with 26 mg (2.88 mmol) of DMAP to the resin. Bubbling was continued at 22 °C for 3 h and the resin was then washed with CH_2Cl_2 (6x15 mL) and dried in vacuo. To test the loading on the resin, 30 mg of resin was removed and

suspended in 2 mL of trifluoroacetic acid for 5 min at 22 °C, filtered and washed (3x3 mL) with CH_2Cl_2 . The filtrate was concentrated in vacuo to give 7.3 mg (85%) of **5**.

Step 2, 16 → 17. A suspension of 690 mg (0.576 mmol) of 2-(9H-fluoren-9-ylmethoxycarbonylamino)-pentanedioic acid 5-allyl ester linked to Wang resin (**16**) in 15 mL of THF was treated with 6 mL (57.6 mmol) of piperidine, agitated by bubbling for 30 min, filtered and washed with CH_2Cl_2 (6X 10 mL). The resin was dried in vacuo. A suspension of this resin in 10 mL of CH_2Cl_2 was treated with 0.48 mL (2.31 mmol) of decanoyl chloride and 14 mg (0.115 mmol) of DMAP. The reaction mixture was agitated at 22 °C for 6 h, filtered and the resin was washed with CH_2Cl_2 (6x10 mL) and dried in vacuo.

Step 3, 17 → 18. A suspension of 690 mg (0.576 mmol) of 2-decanoylamino-pentanedioic acid 5-allyl ester linked to Wang resin (**17**) in 10 mL of THF was treated with 67 mg (0.0576 mmol) of tetrakis(triphenylphosphine) palladium(0) and 806 mg (5.75 mmol) of dimedone, and agitated by bubbling at 22 °C for 18 h. The resin was then filtered, washed with THF (2x10 mL), CH_2Cl_2 (2x10 mL), MeOH (2x10 mL), H_2O (2x10 mL), 1% acetic acid solution (2x10 mL), H_2O (2x10 mL), MeOH (2x10 mL), CH_2Cl_2 (2x10 mL), and dried in vacuo. Cleavage and examination of 40 mg of resin by ^1H NMR showed full deprotection of the allyl ester.

A suspension of this resin in 12 mL of DMF was treated with 0.22 mL (1.572 mmol) of triethylamine and 414.1 mg (2.62 mmol) of Alloc-NHCH₂CH₂NHMe. After agitating the reaction mixture for 5 min to ensure proper mixing, 540 mg (1.572 mmol) of CloP was added. The reaction mixture was agitated with bubbling for 18 h at 30 °C, cooled to 22 °C, and the resin was filtered and washed with DMF (2x10 mL), CH_2Cl_2 (2x10 mL), MeOH (2x10 mL), H_2O (2x10 mL), THF (2x10 mL), and CH_2Cl_2 (2x10 mL). The resin was dried in vacuo and 40 mg of resin was cleaved with $\text{CF}_3\text{CO}_2\text{H}$. The ^1H NMR of the residue showed that coupling had occurred to nearly 100%.

Step 5, 18 → 19. A suspension of 200 mg (0.192 mmol) of 4-[(2-allyloxycarbonylamino-ethyl)-methyl-carbamoyl]-2-decanoylamino-butyric acid linked to Wang resin (**18**) in 6 mL of CH_2Cl_2 was treated with 12 mg (0.0096 mmol) of tetrakis(triphenylphosphine) $\text{Pd}(0)$, 62 μL (0.230 mmol) of tributyltin hydride, and 10 μL of H_2O . The reaction mixture was agitated with bubbling N_2 for 15 min, filtered, and the resin was washed with 10 mL portions of CH_2Cl_2 , THF, acetone, MeOH, H_2O , acetone, EtOAc, hexanes, THF, and CH_2Cl_2 . The resin was then dried in vacuo and 15 mg was removed for testing. The ^1H NMR of the TFA-cleaved residue showed full deprotection as well as full removal of all tin side products.

A suspension of 185 mg (0.190 mmol) of this resin in 8 mL of CH_2Cl_2 was treated with 117 mg (0.576 mmol) of oxazole carboxylic acid, 198 mg (0.576 mmol) of CloP, and 80 μL (0.576 mmol) of triethylamine. The reaction mixture was agitated by bubbling with N_2 for 3 h, filtered, and washed with 20 mL of CH_2Cl_2 , acetone, water, acetone, and CH_2Cl_2 . The resin was dried in vacuo and 15 mg was removed for testing. The ^1H NMR of the residue showed that the reaction had gone to 60% completion. The resin was subsequently submitted to a second coupling cycle.

Step 6, 19 → 1. A suspension of 115 mg (0.12 mmol) of 2-decanoylamino-4-(methyl-{3-[5-methyl-2-phenyl-oxazole-4-carbonyl]-ethyl}-carbamoyl)-butyric acid linked to Wang resin (**19**) in 3 mL of TFA was stirred for 5 min, filtered, and washed with 5 mL of CH_2Cl_2 . The extract was concentrated in vacuo to provide 33.1 mg (100% for step 2 to step 6) of **1**. A ^1H NMR showed the product to be 66% pure with 2-acylamino-pentanedioic acid as the major impurity. Acid **1a** was dissolved in 3 mL of CH_2Cl_2 and treated with 0.016 mL (0.138 mmol) of benzyl bromide and 0.02 mL (0.138 mmol) of DBU to provide material identical with the benzyl ester **2** prepared by solution phase chemistry.

Cell Culture. Human MDA-MB-231 breast carcinoma cells were obtained from the American Type Culture Collection at passage 28 and were maintained for no longer than 20 passages. The cells were grown in RPMI-1640 supplemented with 1% penicillin (100 $\mu\text{g/mL}$) and streptomycin (100 $\mu\text{g/mL}$), 1% L-glutamate, and 10% fetal bovine serum in a humidified incubator at 37 °C under 5% CO_2 in air. Cells were routinely found free of mycoplasma. To remove cells from the monolayer for passage or flow cytometry, we washed them two times with phosphate buffer and briefly (< 3 min) treated the cells with 0.05% trypsin/2 mM EDTA at room temperature. After the addition of at least two volumes of growth medium containing 10% fetal bovine serum, the cells were centrifuged at 1,000 x g for 5 min. Compounds were made into stock solutions using DMSO, and stored at -20 °C. All compounds and controls were added to obtain a final concentration of 0.1-0.2% (v/v) of the final solution for experiments.

PP2A assay. The activity of the catalytic subunit of bovine cardiac muscle PP2A (Gibco-BRL, Gaithersburg, MD) was measured with fluorescein diphosphate (Molecular Probes, Inc., Eugene, OR) as a substrate in 96-well microtiter plates. The final incubation mixture (150 μL) comprised 25 mM Tris (pH = 7.5), 5 mM EDTA, 33 $\mu\text{g/mL}$ BSA, and 20 μM fluorescein diphosphate. Inhibitors were resuspended in DMSO, which was also used as the vehicle control. Reactions were initiated by adding 0.2 units of PP2A and incubated at room temperature overnight. Fluorescence emission from the product was measure with Perseptive Biosystems Cytoflour II (exciton filter, 485 nm; emission filter, 530 nm)(Framingham, MA).

Cell proliferation assay. The antiproliferative activity of newly synthesized compounds was determined by our previously described method.⁴⁶ Briefly, cells (6.5×10^3 cells/ cm^2) were plated in 96 well flat bottom plates for the cytotoxicity studies and incubated at 37 °C for 48 h. The plating medium was aspirated off 96 well plates and 200 μL of growth medium containing drug was added per well. Plates were incubated for 72 h, and then washed 4x with serum

free medium. After washing, 50 μ L of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide solution (2 mg/mL) was added to each well, followed by 150 μ L of complete growth medium. Plates were then incubated an additional 4 h at 37 °C. The solution was aspirated off, 200 μ L of DMSO added, and the plates were shaken for 30 min at room temperature. Absorbance at 540 nm was determined with a Titertek Multiskan Plus plate reader. Biologically active compounds were tested at least 3 independent times.

Measurement of cell cycle kinetics. Cells ($6.5 \times 10^5/\text{cm}^2$) were plated and incubated at 37 °C for 48 h. The plating medium was then aspirated off, and medium containing a concentration of compound **1f** that caused approximately 50% growth inhibition (88-100 μ M) was added for 48 to 72 h. Untreated cells at a similar cell density were used as control populations. Single cell preparations were fixed in ice-cold 1% paraformaldehyde, centrifugation at 1,000 x g for 5 min, resuspended in Puck's saline, centrifuged, and resuspended in ice-cold 70% ethanol overnight. The cells were removed from fixatives by centrifugation (1,000 x g for 5 min) and stained with a 5 μ g/mL propidium iodide and 50 μ g/mL RNase A solution. Flow cytometry analyses were conducted with a Becton Dickinson FACS Star. Single parameter DNA histograms were collected for 10,000 cells, and cell cycle kinetic parameters calculated using DNA cell cycle analysis software version C (Becton Dickinson). Experiments at 72 h were performed at least 3 independent times.

Acknowledgment: Funding was provided by the American Cancer Society (Junior Faculty Research Award to P.W.), Upjohn Co., the Alfred P. Sloan Foundation, and a United States Army Breast Cancer Predoctoral Fellowship. We thank Drs. Boynton and Messner for their preliminary biochemical evaluation of **1a-r**.

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